Thromboelastography

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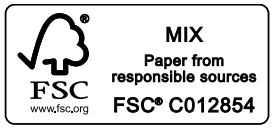
CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Roeloffzen, W.W.H.
Thromboelastography

Proefschrift Groningen. - Met lit. opg. – Met samenvatting in het Nederlands

ISBN: 978-94-6108-273-2
ISBN: 978-90-367-5474-3 (electronic version)
NUGI



Cover: Electromicroscopic image of clot formation.

Lay-out and printed by: Nicole Nijhuis - Gildeprint Drukkerijen - The Netherlands

Financial support for the publication of this thesis was kindly profided by Amgen BV, Bayer BV, Boehringer Ingelheim BV, Celgene BV, Glaxo Smith Kline BV, Janssen-Cilag BV, Mundipharma Pharmaceuticals BV, Novartis Pharma BV, Pfizer BV, Roche Nederland BV, Sanofi BV, Viforpharma BV en de Stichting ter Bevordering van de Haematologie Groningen.

RIJKSUNIVERSITEIT GRONINGEN

Thromboelastography

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 25 april 2012
om 14.30 uur

door

Wilfried Wilhelmus Hendrikus Roeloffzen

geboren op 8 mei 1970
te Wierden

Promotor:	prof. dr. J.C. Kluin-Nelemans
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Laten we van het leven genieten, zolang als we leven

Voor Jasmijn, Merel en Amber

Voor Ingrid

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1

General Introduction and outline of the thesis

INTRODUCTION

Background

The clotting process is a dynamic, highly interwoven array of multiple processes and can be viewed as occurring in several consecutive overlapping stages (1). An interplay between components of the vessel wall, platelets, and coagulation factors give rise to the formation of a haemostatic plug. Several control mechanisms are responsible for modulation and termination of the clotting cascade. Once vessel wall patency has been restored, the mechanism of fibrinolysis is responsible for organizing and removing the formed clot. Classically, laboratory-based coagulation tests (e.g., prothrombin time, activated partial thromboplastin time, fibrinogen) and platelet numbers are being used in evaluating bleeding disorders. However, these tests have several limitations as they are performed in plasma under conditioned circumstances and give fragmented information on mainly the initiation of the coagulation cascade. Moreover, they do not take into account the interaction of clotting cascade and platelets as well as other cellular elements in whole blood (2). Also the influence of hypothermia is not measured, as standard coagulation tests are performed at a standardised temperature of 37˚C. Consequently, complex and multifactorial haemostatic disturbances, as seen in trauma and massive blood loss, are difficult to analyze with these tests. Point-of-care coagulation monitoring devices assessing the viscoelastic properties of whole blood, i.e. thromboelastography may overcome several limitations of routine coagulation tests.

Thromboelastography (TEG) was first described by Hartert in 1948 as a global test of blood coagulation (3). TEG visualizes the viscoelastic changes that occur during coagulation *in vitro* and provides a graphic representation of clot formation and lysis. Clinically, two devices are being used: the TEG® system (Haemoscope Corporation, Niles, Illinois, USA) and the ROTEM (Pentapharm GmbH, Munich, Germany). Although TEG® and ROTEM® traces look similar, the nomenclature and reference ranges are different, and outcomes of both techniques are not interchangeable (4-6). TEG was initially used as a research tool with only limited clinical application. Technical developments and automation some 25 years ago has led to improved

standardization and better reproducibility of the assay. Renewed interest in TEG is also related to the current cell-based model of haemostasis (7). As a global assay of haemostasis performed on whole blood, TEG has the bedside capability to asses within 30 min a representation of the sum of platelet function, coagulation proteases and inhibitors together with the fibrinolytic system, and is therefore considered a helpful coagulation tool in various areas of haemostasis testing by a growing number of physicians.

The main use of TEG has been to monitor blood component therapy during surgery. Its use was first documented in the field of liver transplantation, followed by the field of cardiac- and trauma-surgery (8-11). The use of citrated samples allows TEG to be also performed in the laboratory setting, where it is applied to areas where conventional coagulation testing is considered insufficient (12). These areas include hypercoagulability screening and the assessment of thrombotic risk as well as the guidance of recombinant FVIIa and activated prothrombin complex treatment in patients with haemophilia (13-16). More recently application of a TEG guided transfusion strategy in patients with massive transfusion is advocated in order to reduce the amount of bleeding (17,18).

Principles of TEG® methodology

The basic principle of TEG® involves incubation of 360 µl whole blood at 37˚C in a heated cylindrical cup. The cup oscillates for 10 s through an angle of 4˚ 45’ with a pin freely suspended in the cup by a torsion wire (Figure 1). The torque of the rotating cup is transmitted to the immersed pin only after fibrin-platelet bonding has linked the cup and pin together. The strength of these fibrin-platelet bonds affects the magnitude of the pin motion, such that strong clots move the pin directly in phase with the cup motion. Thus, the magnitude of the output is directly related to the strength of the formed clot. As the clot retracts or lyses, these bonds are broken and the transfer of cup motion is diminished. The rotation movement of the pin is converted by a mechanical-electrical transducer to an electrical signal which can be monitored by a computer program. The resulting haemostasis profile is a measure of time it takes for the first fibrin strands to be formed, the kinetics of clot formation,

the strength of the clot and dissolution of the clot. The clot's physical properties are dependent of the interaction of fibrinogen, platelets and plasma proteins, and this process produces a characteristic trace, reflecting the different phases of the clotting process and enables qualitative evaluation (19) (Figure 2).

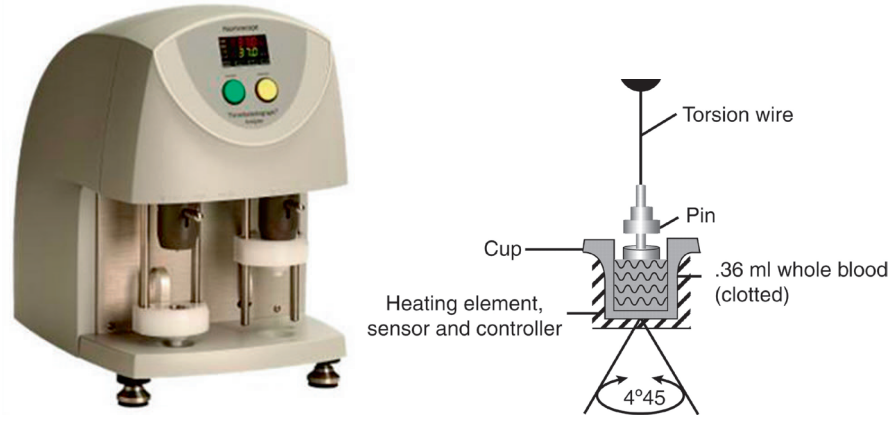

Figure 1.

Thromboelastograph® 5000 coagulation analyzer and basic principles

Five major steps involved in haemostasis are routinely measured by TEG®: The R-time (reaction time) is the latency time from placing blood in the cup until the clot starts to form (taken as reaching a TEG tracing amplitude of 2 mm). The K-time (clotting time) is arbitrary assigned to the time between the TEG trace reaching 2 mm and going up to 20 mm; thus indicating clot kinetics. The alpha angle (α) is a slope drawn from the slope of the TEG tracing from the R to the K value. The alpha angle is visualizing the acceleration and the kinetics of fibrin formation and cross-linking. The maximum amplitude (MA) is the greatest vertical amplitude of the TEG trace and represents clot strength. Clot lyisis at 30 minutes (LY30) measures the rate of amplitude reduction 30 min after MA and provides information on the fibrinolytic activity (Table 1). A fast way to obtain an early impression of the TEG curve is to look at the initial part of the TEG trace represented by the time it takes to reach the maximum amplitude (TMA). The Clot Lysis Time (CLT) is the time measured from MA until the TEG trace finally

reaches the base line at the moment clot lysis is completed. An additional parameter that can be calculated is the shear elastic modulus strength or clot elasticity (SEMS or G, dynes cm^{-2}), which is a parametric measure of clot firmness, expressed in metric units, calculated from MA as follows: G = (5000 x MA) / (100 - MA). R-time, K-time and α are prolonged by anticoagulants and coagulation factor deficiencies, MA is especially influenced by platelet count and platelet function as well as fibrinogen level.

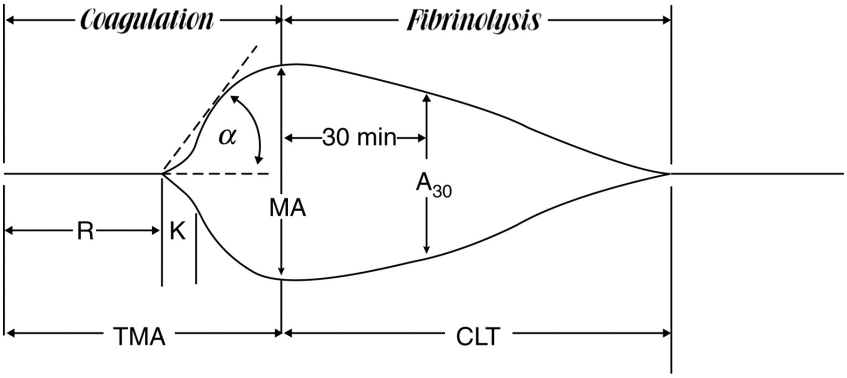

Figure 2.

Thromboelastograph® trace and parameters

Variations on the TEG®

Coagulation *in vitro* is usually initiated by the addition of calcium to citrated plasma. However, for TEG analysis, use of native whole blood is more reliable and considered the gold standard (20). If rapid information is required, the blood sample can be activated by the addition of celite or tissue factor, both causing shortening of the R-time. The effect of heparin can be evaluated by placing the blood samples into cups that have been coated with the enzyme heparinase (21). With the Reopro-modified TEG assay the contribution of fibrinogen to clot strength (TEG parameter MA) can be evaluated (22). The effects of glycoprotein IIb/IIIa inhibitors, acetylic acid or ADP agonists can be visualized by comparing standard and modified ("platelet mapping") TEG curves (23). Finally, the TEG® haemostasis system is able to produce

a thrombus generation velocity curve, which can be calculated from the initial part of the TEG trace by differentiating the entire initial time-course, providing three new parameters of thrombus generation (24).

Table 1. Thromboelastograph® parameters and corresponding measurements

	TEG parameter measured:	Influenced by / reflecting:
R	R-time is the period of time of latency from the time that the blood was placed in the TEG until the initial fibrin formation.	prolonged: - anticoagulants shortened: - hypercoagulable conditions
K	K-time is a measure of the speed to reach a certain level of clot strength.	prolonged: - anticoagulants shortened: - increased fibrinogen (- increased platelet function)
α	Alpha angle measures the rapidity of fibrin build-up and cross-linking (clot strengthening).	decreased: - anticoagulants increased : - increased fibrinogen (- increased platelet function)
MA	MA, or Maximum Amplitude, is a direct function of the maximum dynamic properties of fibrin and platelet bonding via GPIIb/IIIa and represents the ultimate strength of the fibrin clot.	reflecting : - platelet count & function - fibrinogen level
Ly30	LY 30 measures the rate of amplitude reduction 30 minutes after MA.	reflecting : - fibrinolytic activity

Outline of the thesis.

As stated in the introduction, TEG is gaining popularity as a rapid near site global test of haemostasis in different clinical areas. In contrast to standard coagulation tests (performed in the central laboratory), the technique is considered easy to perform, with results that are easy interpretable and have direct consequences for clinical practice. However, although TEG poses many (theoretical) advantages over classical coagulation tests, clinicians should be aware of pitfalls in both test methodology as well as its interpretation. Importantly, studies comparing TEG parameters with standard coagulation tests demonstrate poor correlation and there are no clinical trials that link TEG variables to clinical outcome. The overall aim of this thesis is to improve the understanding of TEG technology for clinicians working in the field of haemostasis, making them aware of the possibilities but also of the limitations of TEG in coagulation monitoring.

In *Chapter 2* we describe, based on literature study, the role TEG and other Point of Care tests of haemostasis can play in both the prediction and treatment of unexpected massive surgical bleeding. For this purpose we will outline the actual cell-based model of haemostasis, with special focus on the haemostatic effects of both massive blood loss and massive transfusion. Secondly we will concentrate on what part of the coagulation cascade exactly can be monitored with which specific coagulation test. Finally we discuss the possibilities and limitations of these Point of Care tests of haemostasis in the analysis of the massively bleeding patient, with the purpose of improving the use of these tests in the clinical analysis and decision making in this patient category.

As the interpretation of TEG data is hampered by the lack of a validated large series of reference ranges, with special attention for the effects of age, gender and the use of oral contraceptives, we assessed our own reference ranges, for both native whole blood and recalcified citrated whole blood, which are presented in *Chapter 3*. Importantly, we used these reference ranges for comparison in the other studies that we performed.

Different components of the TEG test tracing are considered to reflect various parts of the haemostatic system and to distinguish low platelet count and/or platelet dysfunction from lack of plasmatic coagulation factors. In *Chapter 4* we describe the effects of (isolated) thrombocytopenia on TEG parameters and discuss the sensitivity of TEG in the detection of (clinical relevant) thrombocytopenia. For this purpose we performed TEG measurements serially in patients with well documented transient thrombocytopenia in the context of consolidation courses of chemotherapy because of hematological malignancy.

As we hypothesized that TEG might be a useful tool to monitor the haemostatic quality and efficacy of platelet transfusions we describe in *Chapter 5* the results of our study in which the haemostatic effects of transfused stored platelets were compared to native circulating platelets, as measured by TEG. In this study we also assess whether storage time of platelets has additional influence on their haemostatic capacity.

In *Chapter 6* we used TEG to study the role of red blood cells in haemostasis. For this purpose we studied haemostasis in patients with different degrees of anaemia. Moreover, we studied the effects of red blood cell transfusion on the haemostatic profile, with again attention for effects of storage time of the red blood cell product.

In line with *chapter 6* is the study described in *Chapter 7*, in which we address the coagulation profile in patients with sickle cell disease, as we hypothesized that TEG might be a useful tool to analyze the pro-coagulant profile observed in this patient category. Further we examined the differences in coagulation status between steady state and painful vaso-occlusive crises in these patients. Moreover, we also focused on the effect of red and white blood cells as well as treatment with Hydroxyurea on this status.

In *Chapter 8* we will present a summary and future perspectives on the role TEG can play in haemostasis testing.

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2

Application of Point-Of-Care testing of haemostasis in massive blood loss; possibilities and limitations

Wilfried W.H. Roeloffzen, Hanneke C. Kluin-Nelemans, Joost Th.M.de Wolf

Submitted

ABSTRACT/SUMMARY

The management of massive blood loss remains a major challenge for clinicians as the underlying coagulopathy is often complex, multifactorial and rapidly evolving. The use of classical coagulation tests, although standardized and well validated, has limitations as these tests are time consuming and provide only information on isolated coagulation factors. To overcome several of these limitations, point-of-care (POC) tests of haemostasis have been introduced in various areas of coagulation monitoring. POC tests that asses global haemostasis, e.g. thromboelastography, have potential for allowing a new look at the process of haemostasis and might add to the management of massive blood loss, when incorporated into a transfusion algorithm. As these POC tests become increasingly used outside of the specialised laboratory, attention should be paid to validation, reliability and quality control testing as several techniques are not validated and have never been standardized. The present review gives an overview on the current cell-based model of haemostasis and the pathophysiology of massive blood loss. Further we describe the different POC analysers and methods available and give insight into which parameters of the coagulation cascade can be monitored best by these techniques and for which clinical indications. Moreover, we will focus on the clinical relevance of the POC test in the prediction of surgical bleeding as well as the analysis of unexpected massive blood loss.

INTRODUCTION

The detection, analysis and correction of haemostatic defects in the patient with massive blood loss in the operating theatre remains a major challenge for clinicians. Defects in haemostasis may complicate a wide range of surgical conditions and much attention has been paid to the management of massive blood loss in patients undergoing cardiac, hepatic and trauma surgery, all associated with complex and multifactorial coagulopathies (1-3). Although still the standard in haemostasis testing, laboratory-based coagulation tests are considered to have several limitations in haemorrhagic emergencies. First of all, classical coagulation tests are considered time consuming as they are performed in a central laboratory, leading to a delay in diagnosis and management of the coagulopathy (4). Further, these classical tests provide only a snapshot of the coagulation status on the moment the sample was taken, as these tests are based on isolated static end points (5). Also the influence of hypothermia is not measured as classical coagulation tests are performed in plasma at a normal and standardised temperature of 37˚C. Moreover, although the classical view of the clotting cascade has been useful in the interpretation of clotting times (e.g., PT and aPTT), it may not be physiologically accurate (6). Finally, the correlation of laboratory-based coagulation tests with clinical bleeding is currently imprecise and requires a more rapid evaluation of haemostasis and fibrinolysis (7). As a consequence, “bedside” performed point-of-care (POC) testing of haemostasis is increasingly being incorporated in the management of massive blood loss in the operating theatre (8,9). Arguments given in favour of POC tests of haemostasis are the rapid, real-time information these tests provide on the coagulation process (10). Moreover, visco-elastic POC testing i.e. tromboealastography, is considered more physiological than classical coagulation testing as it is performed on whole blood, taking into account the interaction of the coagulation system with all other cellular elements (11).

Since POC testing of haemostasis has gained popularity, it may be tempting for clinicians to replace classical coagulation tests by these rapid and ”easy interpretable” POC tests in all patients suffering from massive unexpected blood loss in the operating

theatre. However, before such a change in paradigm can take place, there are several aspects of POC testing that need to be ascertained. The most important question to be answered is whether POC tests are really suited to monitor massive blood loss in general surgery. Secondly, is there sufficient evidence to fully support its use in the management of massive unexpected blood loss? To answer the former questions, we can speculate on what the "ideal" POC test of haemostasis looks like and what the requirements of such a test are (12). Ideally a POC test should deliver a timely result that is precise with adequate quality and has the potential to improve patient outcome from earlier treatment. As a consequence the test should be performed by skilled and trained personal, aware of the tests' critical analytical steps and its potential errors and limitations (13). Further, the technique should be standardized according to international guidelines and be subjected to strict internal- and external-quality control procedures (14,15). Moreover, the POC test should be validated, not only for detection and analysis of the different coagulation disorders, but also for monitoring the effects of both anticoagulants and haemostatic agents. Also reference ranges should be established in different age- and gender- groups. Importantly, the test should be subjected to randomized controlled trials demonstrating positive effect on patient care. Finally, the POC data should be stored and transmitted to a POC data manager or hospital information system. The most important aspects and requirements of the "ideal" POC test of haemostasis are summarized in Table 1.

Table 1. The "Ideal" POC test of Haemostasis

Important Aspects and Requirements of the "Ideal" POC test.
• Generation of rapid and real-time results and so earlier treatment
• Knowledge of critical analytical steps and potential errors
• Trained and skilled operators
• Standardization of the technique according to international guidelines
• Internal and external quality control and assurance
• Validation of technique for different coagulopathies and monitoring of anticoagulants and haemostatic agents
• Reference ranges for different clinical conditions, age and gender
• Data storage and transmittance to hospital information system
• Randomized controlled trials demonstrating positive effects on patient outcome

Scope of this review

Advanced coagulation monitoring in massive blood loss might employ a combination of routine laboratory tests using individual factor measurements as well as POC coagulation testing. Therefore, we consider it valuable for clinicians using these tests and techniques, to have knowledge of the possibilities and limitations of the different POC tests of haemostasis. For this purpose we will first (briefly) overview the current (cell-based) model of haemostasis, with special focus on the coagulopathy that arises in massive bleeding. Subsequently we will update and review the POC analysers and methods available and give insight into which parameters of the coagulation cascade exactly can be monitored by these techniques and in which clinical condition. Moreover, we will focus on the clinical benefit the POC test has in the prediction of surgical bleeding as well as the analysis of massive blood loss in the operating theatre. The final goal is that clinicians, overseeing all the possibilities and limitations of POC testing of haemostasis, can make a well balanced decision on the appropriate POC test of haemostasis to be implemented in their operating theatre.

Principles of haemostasis

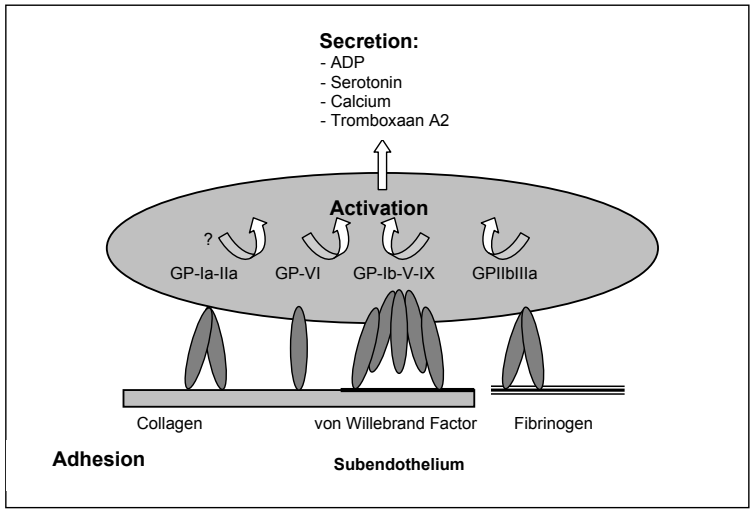
Although the clotting process is a dynamic, highly interwoven array of multiple processes, it can be viewed as occurring in several consecutive overlapping stages (16). An interplay between components of the vessel wall, platelets, and coagulation factors give rise to the formation of a haemostatic plug. Several control mechanisms are responsible for modulation and termination of the clotting cascade. Once vessel wall patency has been restored the mechanism of fibrinolysis is responsible for organizing and removing the formed clot.

• *Primary haemostasis; platelet adhesion and aggregation*

Platelets play an important role in localizing clotting reactions because they adhere and aggregate at the sites of injury where tissue factor (TF) is also exposed. They provide the primary surface for generation of the burst of thrombin needed for effective haemostasis during the propagation phase of coagulation. Platelet localisation and activation are mediated by Von Willebrand Factor (VWF), thrombin, platelet receptors, and vessel wall components, such as collagen (17). Circulating

platelets get in close contact to the injured vessel wall by attachment of the platelet surface Glycoprotein Ib (GP-Ib) to VWF in the wound (Fig. 1). VWF is a large molecule, synthesized by endothelial cells, and also found in granules of platelets, that circulates in complex with coagulation factor VIII (FVIII). After binding to the exposed collagen in the wound, VWF is structurally changed and able to bind GP-1b on the platelet surface.

Figure 1. Platelet adhesion, activation and secretion in primary haemostasis



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During high shear, platelets "role" along the wound surface and, initially, detach, until they lose their speed and bind irreversibly to the wound surface. The binding is further increased by attachment of both GP-Ia-IIa complex and GP-VI to collagen; which results into platelet activation. Moreover, when fibrinogen and fibrin are attached to the wound surface, platelets bind via their GP-IIb-IIIa receptor. Platelet adhesion is followed by platelet activation mediating platelet spreading and clot retraction. Also upon activation, platelets release a variety of substances from their granules like ADP, serotonin and tromboxane A2, stimulating and recruiting additional platelets.

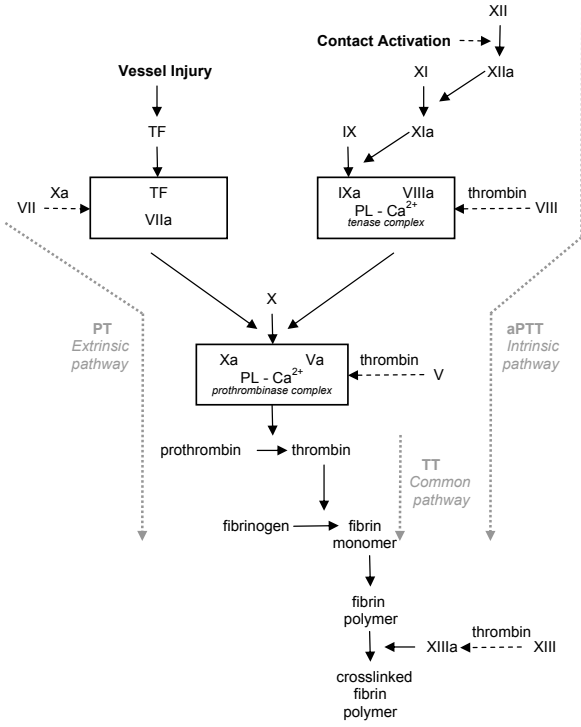
Parallel to adhesion, aggregation and secretion of activated platelets, haemostasis is further enhanced by formation of thrombin which further activates platelets. Several platelet-blocking agents can interfere with primary haemostasis. Aspirin and other non-steroidal anti-inflammatory agents inhibit cyclo-oxygenase (COX), the enzyme responsible for the formation of tromboxane A2 out of arachidonic acid (AA) from membrane phospholipids. The thienopyridine derivates (ticlopidin, clopidogrel) inhibit ADP-induced platelet activation by binding covalently to the P2Y12 receptor. Finally, GP-IIb-IIIa antagonists (abciximab, tirofiban, eptifibatide) block GP-IIb-IIIa receptors.

• *Secondary haemostasis; initiation, amplification and propagation of the clotting cascade*

The generation or exposure of TF at the wound site, and its interaction with FVII, is the primary physiologic event in initiating clotting (18). The initiation process takes place on the surface of TF-bearing cells such as fibroblasts and platelets where FVII binds to TF and is activated. This FVII/TF complex activates Factor X (FX) which in turn generates a small amount of thrombin that plays a role in the amplification stage (Fig. 2). Thrombin is generated by a complex network of amplification and negative feedback loops to ensure a localized and limited production.

The generation of thrombin is dependent on three enzyme complexes, each consisting of a protease, a cofactor and catalyzed by phospholipids (PL) and calcium (Ca2+). These three enzyme complexes are extrinsic Xase (FVIIa, TF, PL, Ca2+), intrinsic Xase or tenase complex (FIXa, FVIIIa, PL, Ca2+) both generating FXa, and prothrombinase complex (FXa, FVa, PL, Ca2+) generating thrombin. With sufficient stimulus, components of the intrinsic pathway (i.e., factors VIII, IX, XI) are responsible for amplification of this process only after a small initial amount of thrombin has been generated through the extrinsic pathway (19). Moreover, once platelets are activated, the cofactors Va and VIIIa are rapidly localized on the platelet surface (20). The propagation phase catalysts (FVIIIa-FIXa and FVa-FXa) continue to drive the reaction as blood is resupplied to the wound site by flow. The generation of thrombin via the coagulation cascade results in deposition and cross-linking of fibrin. Ultimately, the net result of platelet aggregation, fibrin deposition, and fibrin cross-linking, is the formation of a stable clot.

Figure 2. Overview of the coagulation cascade



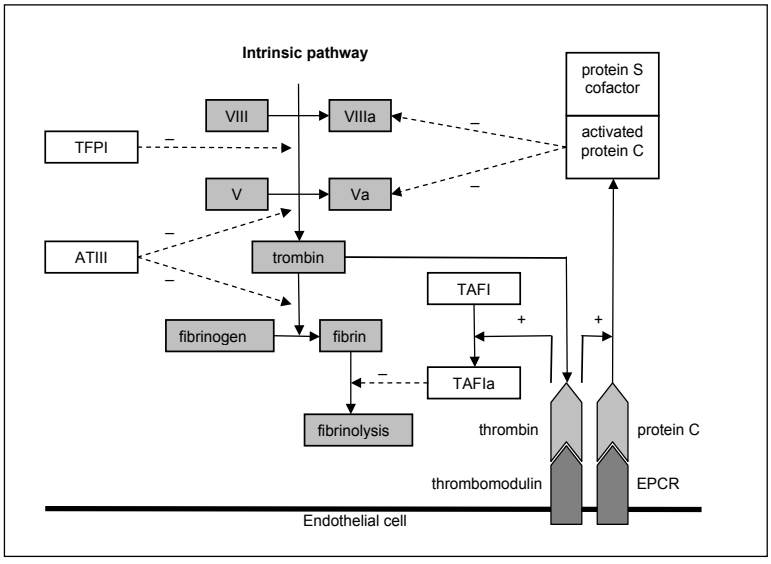
PT; prothrombin time, aPTT; activated partial thromboplastin time, TT; thrombin time, TF: tissue factor, PL; platelets, Ca^{2+}; Calcium

• *Terminating of clotting by antithrombotic control mechanism*

The control of the haemostatic reaction is governed by the pro- and anticoagulant dynamics and the supply of blood reactants to the site of a vessel injury (21). Termination of coagulation is regulated by the plasma proteins tissue factor pathway inhibitor (TFPI), antithrombin (AT), protein C and protein S. The initiation phase (extrinsic Xase) is rapidly inactivated by TFPI, released form platelets and endothelial cells as it forms a quaternary complex with FVIIa, TF and FXa, such that only a limited amount of thrombin is generated. Thrombin forms a complex with the endothelial cell surface receptor thrombomodulin. An endothelial protein C receptor (EPCR) localizes protein C to the endothelial surface, promoting protein C activation by the thrombin-

thrombomodulin complex. Protein C (and its cofactor Protein S) are inhibitors of coagulation factors Va and VIIIa, preventing further thrombin generation. Finally, the thrombin-thrombomodulin complex is also the activator of the fibrinolysis inhibitor TAFI (Fig. 3). One of the major natural inhibitors of thrombin is AT, which is only active when coagulation is activated as it only binds thrombin, and not prothrombin. Moreover, AT binds to all activated coagulation factors with serine proteases (FIXa, FXa, FXIa and the FVIIa-TF complex) to form high molecular weight stable complexes. Importantly, the reaction between thrombin and AT can be accelerated by heparin.

Figure 3. Limitation of blood coagulation



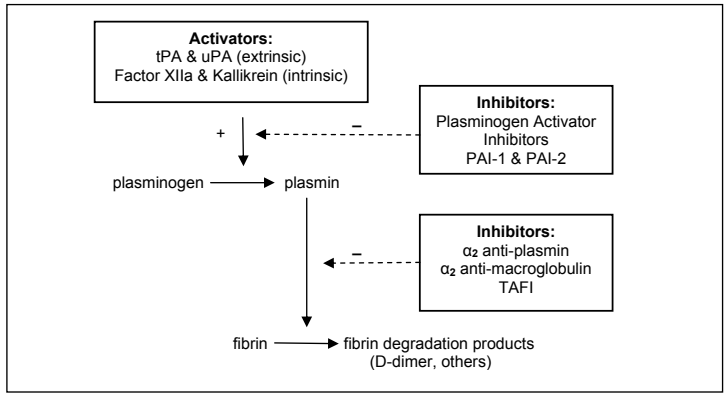
TFPI; tissue factor pathway inhibitor, ATIII; antithrombin, TAFI; thrombin-activatable fibrinolysis inhibitor; EPCR; endothelial protein C receptor

• *Removal of the clot by fibrinolysis*

Fibrinolysis is a normal haemostatic response to vascular injury. The final effector of fibrinolysis is plasmin, produced from the inactive precursor plasminogen by activators either from the vessel wall (intrinsic activation) or from the tissues (extrinsic activation) (Fig. 4). The most important route follows the release of tissue

plasminogen activator (tPA) from endothelial cells. tPA and plasminogen both bind to the evolving fibrin polymer. Once plasminogen is activated to plasmin it cleaves fibrin into soluble fibrin degradation products (FDPs) resulting in dissolution of the fibrin clot. One of the major FDPs is D-dimer, which consists of two D domains from adjacent fibrin monomers that have been crosslinked by activated FXIII.

Figure 4. Fibrinolysis



tPA; tissue Plasminogen Activator, uPA; urokinase like Plasminogen Activator, PAI-1&-2; Plasminogen Activator Inhibitor 1 & 2, TAFI; Thrombin-Activated Fibrinolysis Inhibitor

Coagulation stabilization counteracts fibrinolysis through several mechanisms. FXIIIa converts fibrin into a tight-knit aggregate. tPA is inactivated by plasminogen activator inhibitors (PAI); PAI-1 is a rapid and irreversible inhibitor of tPA and uPA (urokinase like plasminogen activator). Further, circulating plasmin is inactivated by the potent inhibitors alpha-2 antiplasmin and alpha-2-macroglobin (22). Thrombin inhibits fibrinolysis by activating TAFI that removes lysine residues from fibrin, thereby impairing fibrin's capacity to bind plasminogen and tPA.

Coagulopathy in massive bleeding

Haemostasis is the process of quick, localized and carefully regulated clot formation at the site of vessel injury. An optimal pH value and body temperature as well as adequate quantities of ionised calcium are relevant pre-conditions for optimal haemostasis.

Abnormal bleeding may occur when specific elements of the process are missing or dysfunctional, for example in case of Haemophilia or severe thrombocytopenia. Also the use of anticoagulants can be the cause of abnormal bleeding. During massive bleeding (-and transfusion), with loss of coagulation factors, the evolving coagulopathy is often complex and multifactorial and aggravated by hypoperfusion, dilution- and consumption- of clotting factors and platelets. This coagulopathy, together with hypothermia and acidosis, forms a "lethal" triad (23).

Dilutional coagulopathy is observed in patients who have lost blood and had the blood volume replaced with components without plasma procoagulants and anticoagulants, i.e. if volume replacement is mainly performed with red cells, crystalloid and plasma expanders (24). Mild dilution mainly results in reduction of clot firmness, this being significantly more reduced with colloids than with crystalloids. With dilution >50% a delay in the initiation of coagulation is observed (25). Synthetic colloid resuscitation fluids (i.e. hydroxyethyl starch; HES) induce a coagulopathy that is characterised by acquired hypofibrinogenaemia and abnormal fibrin polymerization (26). Fibrin polymerization is also disturbed following extensive administration of blood products (platelets and plasma) as these fractionated blood products are stored in preservative solutions containing citrate causing hypocalcaemia (27). Fibrinogen may become critically low in massive bleeding with a fall below 1 g/l after a loss of 150% of the calculated blood volume (28). Coagulation factors II, V and VII as well as platelet levels become critical after a blood volume loss of more than 200% (29). Some patients with massive bleeding are also at risk of consumptive coagulopathy and are liable to develop haemostatic failure without significant dilution. Consumption of coagulation factors is seen in obstetric haemorrhage, in patients on cardiopulmonary bypass (CPB), following massive trauma and in the context of sepsis (30). In addition, massive trauma and shock are associated with activation of anticoagulant pathways, especially the thrombomodulin-protein C pathway, resulting in increased fibrinolysis (31).

On top of dilution and consumption of coagulation factors, hypothermia and acidosis may worsen the developing coagulopathy. Hypothermia-induced coagulopathy

attributes to platelet dysfunction, reduced coagulation factor activity (significant below 33˚C) and induction of fibrinolysis (32). For each 1˚C decline in body temperature, there is a 10% reduction in the coagulation factor activity (33). In clinical practice, the effect of hypothermia on coagulation is likely to be underestimated as most coagulation tests are performed at 37˚C thereby correcting ex vivo any in vivo abnormality (34). Acidosis is induced by hypoperfusion and excess administration of ionic chloride, i.e. NaCl, impairing almost all essential parts of the coagulation process (2). Acidosis not only impairs platelet function as well as the activity of coagulation factor complexes and the generation of thrombin, but also leads to an increased degradation of fibrinogen, especially at a PH <7.1 (35,36). Although correction of hypothermia reverses its detrimental effects on coagulation, correction of acidosis by administration of buffer solutions has no such effect (37).

Laboratory-based coagulation tests

• *Tests of primary haemostasis; platelet count and function*

As platelets play an essential role in (primary) haemostasis, the accurate measurement of their number and function is important. Laboratories quantify platelet counts by automated cell analyzers that mostly are reliable at even very low platelet levels (38). However, this method is not perfect as in vitro micro-aggregation and EDTA-induced aggregation may lead to underestimation of the absolute platelet count. This EDTA-dependent pseudo thrombocytopenia can be ruled out by recounting platelets in citrated blood. A platelet count <50x109/l in a patient with massive blood loss is associated with haemostatic compromise and microvascular bleeding. A minimum target platelet count of 75x109/L is appropriate in this situation.

Platelet function is traditionally tested for by performing a bleeding time. However, for bleeding time there is a lack of standardisation, the test is invasive, subjective and has low sensitivity and specificity in the detection of mild bleeding disorders (39,40). Moreover, the most common cause of bleeding time prolongation is improper performance of the test and only experienced laboratory personnel should perform it. Importantly, no studies have been performed to evaluate the role of bleeding time in patients with massive bleeding. However, in patients with a negative history

of bleeding and no recent intake of non-steroidal anti-inflammatory drugs, higher values for bleeding time and bleeding time-related parameters were not associated with higher indices of perioperative and postoperative bleeding at coronary bypass surgery (41).

Traditional assays, such as light transmittance aggregometry, are still considered as the gold standard for platelet function testing. In this assay, platelet agonists are added to platelet rich plasma and the increase of light transmittance is recorded as platelets start to aggregate. Only one study, performed in patients undergoing off-pump coronary bypass grafting demonstrated that intraoperative bleeding was significantly correlated with the decrease in the pre- vs. postoperative platelet count and function according to the area under the impedance curve using whole blood aggregometry. However, no effects on the use of blood products was observed (42). Aggregation will remain an important clinical test within the specialised laboratory. Many platelet disorders are easily diagnosed, but the technique has several limitations, as the preparation of specimens is difficult, time consuming, operator dependent and it cannot be performed at the bedside (43). As a consequence, the test is considered too complex and impractical to use in the management of patient with massive blood loss.

• *Tests of the coagulation cascade; aPTT, PT and TT*

The in vitro tests activated partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin time (TT) measure the time elapsed from activation of the coagulation cascade at different points to the generation of fibrin (Fig. 2). Citrated plasma, an activator (tissue factor for PT and phosholipids for aPTT) are added together and incubated at 37˚C. Calcium is added and the time necessary for clot formation is measured. The aPTT is used to assess the contact activation and the integrity of the intrinsic coagulation pathway (factors XII, XI, IX, VIII) and final common pathway (factors II (=prothrombin), V, X, and fibrinogen). Prolonged aPTT can be found due to isolated deficiencies (or inhibitors) of intrinsic- and common- pathway factors, and after heparin administration. Artificially prolonged aPTT occurs in case of underfilling the test tube, improper blood collection, and with elevated haematocrit.

High concentrations of heparin (>1.0 U/ml), as employed in coronary surgery, can be monitored by the activated whole blood clotting time (ACT), since aPTT often becomes infinitely prolonged at these higher concentrations (44). The aPTT is often used to guide blood product replacement with a target aPTT below 1.5 times normal (<60 s). However, the latter may not always be an appropriate target in the situation of massive blood loss as haemostatic failure may already be significant at this level (9).

The PT is used to assess the integrity of the extrinsic pathway of clotting, which consists of TF and FVII and coagulation factors in the common pathway. The function of PT has been standardized (for warfarin control) through the use of the international normalized ratio (INR). Causes of isolated prolonged PT are inherited or acquired FVII deficiency, VKA administration, vitamin K deficiency, liver disease and an inhibitor of FVII. PT is not always a sensitive test for the actual coagulation status as a normal PT does not rule out haemostatic abnormalities. In massive blood loss a correction to within 1.5 of normal is advocated, although this may not be an appropriate target in this clinical situation (45).

When both PT and aPTT are prolonged inherited deficiency of prothrombin, fibrinogen or factors V or X can be the cause. More often there is an acquired cause of these prolongations, i.e. liver failure, disseminated intravascular coagulation, overdosing with heparin or VKA or the combination of both. Finally an inhibitor of prothrombin, fibrinogen or factors V or X of the common pathway can be the cause of combined prolongation of PT and aPTT.

Both pathways (intrinsic and extrinsic) converge on the activation of Factor X, which converts prothrombin to thrombin, the final enzyme of the clotting cascade, which in turn converts fibrinogen into fibrin. The Thrombin Time (TT) measures this final step of the coagulation cascade (46). Prolongation of the TT can be explained in three ways: deficient fibrinogen (<100 mg/dl), abnormal fibrinogen (dysfibrinogenemia), or an inhibitor to its activation. Acquired deficiency of fibrinogen is usually due to a consumptive coagulopathy. Severe liver disease is associated with both acquired

deficiency of fibrinogen and dysfibrinogenemia. The most common acquired inhibitors of the final reaction are heparin and fibrin degradation products (FDP). The effect of heparin can be eliminated by catalyzing the reaction with reptilase, which, unlike thrombin, is insensitive to heparin. FDP are commonly seen in consumptive coagulopathies and primary fibrinolytic states.

• *Fibrinogen concentration.*
In the conventional Clauss method, diluted plasma is mixed with a supraphysiologic amount of thrombin; the fibrinogen concentration is proportional to the coagulation time measured. The formation of fibrin is a key step in blood coagulation, and hypofibrinogenemia is a usual component of complex coagulopathies associated with massive bleeding. Fibrinogen plays a major role in routine coagulation tests like PT and aPTT. Moreover, the fibrinogen level is more sensitive than the PT and aPTT to a developing dilutional or consumptive coagulopathy. Excessive bleeding has been reported at fibrinogen levels below 0.5-1.0 g/L; levels above 1.5 g/L are probably required for sufficient fibrin clot polymerisation (47).

POC based coagulation tests
A number of coagulation tests can be performed at the point-of-care, rather than in a central laboratory, with the rationale that this will result in the rapid generation of results and will improve patient care. POC testing may measure platelet function, PT, aPTT, INR, ACT, D-dimer as well as overall coagulation (48). POC tests are also being used to diagnose acquired or inherited coagulopathy, to monitor anticoagulant therapy and to guide transfusion of blood and blood products (49,50). A summary of POC tests and devices available for haemostasis monitoring and their clinical use is presented in Table 2.

In the following section we will discuss several important aspects of these POC tests and techniques. First we will briefly outline the principle of each test and address its clinical use and limitations, especially in the context of the patient suffering from massive blood loss. As d-dimer assays are mainly used in the analysis of venous thromboembolism, we will consider this out of the scope of this review.

Table 2. Overview on POC tests of haemostasis

POC Test ***(Aspect of haemostasis tested)***	**Devices**	**Clinical use**
Platelet Function Tests *(primary haemostasis)*	PFA-100® Rapid Platelet Function Assay (RPFA) Plateletworks® Multiplate Verify Now Thromboelastography; plateletmapping	platelet count, detection of platelet dysfunction & inhibition, guidance on therapeutic intervention (i.e. in cardio-vascular surgery)
Prothrombin Time & International Normalized Ratio *(extrinsic pathway)*	Coagucheck (X)S plus Hemochron Response INRatio Monitoring System Protime 3 i-Stat Gem PCL Cascade POC Thrombotrak	control on oral anticoagulation (vitamin K antagonists), assessment extrinsic pathway of coagulation
Activated Partial Thromboplastin Time *(intrinsic pathway)*	Hemochron Junior Signature Gem PCL Cascade POC	heparin monitoring, assessment intrinsic pathway of coagulation
Activated Clotting Time *(intrinsic pathway)*	Hemochron Jr ACT Gem PCL i-STAT Actalyke MAX-ACT Hepcon Haemostasis Management System (HMS)	high dose heparin monitoring
Thrombin Time (fibrinogen→ fibrin)	Hemochron HiTT	high dose heparin monitoring
Fibrinogen	Hemochron Signature Elite	fibrinogen level
Thrombelastography *(global assessment of haemostasis)*	TEG® ROTEM®	detection of coagulopathy, guidance on therapeutic intervention
D-dimer *(fibrinolysis/ degradation of clot)*	Clearview Symplify D-dimer Cobas h232 system SimplyRed D-dimer MiniQuant Nycocard D-dimer Triage D-Dimer Test Vidas Pathfast	exclusion of Venous Thromboembolism (VTE)

• *POC tests on Platelet Function.*

The assessment of bedside platelet function has gained attention in two areas of coagulation monitoring. First of all, platelet function testing devices have been developed to monitor anti-platelet therapy in cardiovascular patients, especially during cardiopulmonary bypass surgery (51). Second, visco-elastic POC coagulation devices can be used to facilitate the management of bleeding patients by rationalizing platelet transfusion (52). A variety of devices are available that offer POC testing of platelet function (see for overview Table 3).

Platelet Function Analyser (PFA-100). The PFA-100 measures the closure time i.e. the time to cessation of flow of citrated blood, flowing at high shear rate through an aperture within a capillary membrane impregnated with the agonists collagen and either epinephrine or adenosine diphosphate (53,54). The test is simple and rapid, does not require specialist training and may serve as a screening tool for platelet dysfunction, in particular as seen in Von Willebrand disease (55,56). However, test outcome is not specific for any other thrombocytopathy (57). In the assessment of the response to anti-platelet therapy the PFA-100 was found to be sensitive to the effects of aspirin, but relatively insensitive to the effects of clopidogrel (58,59).

Although a normal ADP stimulated closure time was found to be a negative predictor of bleeding (60), it was also demonstrated that is was less accurate than classical ADP-mediated platelet aggregometry in risk stratification of perioperative bleeding and transfusion (61). A major limitation for the use of the PFA-100 as a POC test of primary haemostasis in the (massive) bleeding patient is it's sensitivity to thrombocytopenia (<100x10^9/l) and anaemia (haematocrit <30%) both resulting in prolongation of the closure time (62). Moreover, there is only limited literature on whether an exact threshold closure time exists above which bleeding tendency is increased and below which invasive interventions can be performed safely.

Table 3. POC tests of platelet function

POC test	Principle	Clinical Use	Limitations
PFA-100®	high shear adhesion & aggregation of platelets, after agonist, forming a plug, expressed as Closure Time (CT)	• rapid diagnosis of: - von Willebrand disease - generalized platelet dysfunction	• thrombocytopenia (<100x10E9/L) & anaemia (haematocrit <35%): ↑ of CT • not specific for any disorder; just screening • wide normal range • low sensitivity detecting storage and release defects
RPFA (VerifyNow® systems)	optical aggregometry of platelets over time in response to agonists that are specific to various anti-platelet medications.	• measurement of platelet responsiveness to anti-platelet medications (aspirin, clopidogrel, abciximab)	• not performed under shear stress • high costs of cartridges • inconsistency over time in identification of aspirin-resistance
PlateletWorks®	comparing platelet counts before and after aggregation with agonist	• platelet count with an evaluation of platelet function • detection of resistance to antiplatelet drugs	• collagen cartridge not recommended for assessment of aspirin
Multiplate®	impedance aggregometry	• detection of clodipogrel resistance • assesment of need for transfusion in cardiac surgery	• Reference ranges vary widely • ADP aggregation: 30 min. pre-analytical equilibration • not validated for low platelet counts

Because of the inconsistencies in literature, the Platelet Physiology Subcommittee of the Scientific and Standardization Committee of The International Society on Thrombosis and Haemostasis (ISTH-SSC) states that the PFA-100 closure time should be considered optional in the evaluation of platelet disorders and function, and that its use in therapeutic monitoring of platelet function is currently best restricted to research studies and prospective clinical trials (63).

The Rapid Platelet Function Assay (RPFA; VerifyNow® systems). The VerifyNow® System measures platelet function by the rate and extent of light changes in whole

blood as platelets aggregate over time in response to agonists that are specific to various anti-platelet medications such as GPIIb/IIIa inhibitors (abciximab), aspirin and P2Y12 antagonists (64). The VerifyNow assay is simple to handle, gives rapid results and can be used at the patients point-of-care. The assay is mainly applied in cardiovascular patients to monitor the effects of (-or resistance against) platelet antagonists. The VerifyNow results correlated with clinical outcome in patients treated with respectively GPIIb/IIIa inhibitors, aspirin and P2Y12 antagonists (65-67). A limitation is that the test is not performed under shear stress. Further, a lack of consistency over time in the identification of aspirin resistance was found by others (68). Therefore, until so far, the VerifyNow assays are still considered research tools, not yet suitable for guiding clinical decision making, until prospective trials prove otherwise (69,70). As a consequence, this test of platelet function has no role in analyzing and treating coaugulopathy in patients with (massive) bleeding.

Plateletworks®. Plateleworks (PW) is a simple and easy to use whole blood assay that compares platelet counts in a control tube with platelet counts in a citrate tube after aggregation with either ADP or collagen (71). The PW ADP cartridge appears useful for the assessment of both P2Y12 inhibitors and GPIIb/IIIa antagonists (72). In patients undergoing cardiopulmonary bypass surgery a correlation was found between PW collagen reagent tubes preoperatively and chest tube drainage and was considered a useful predictor of blood product use (73). In another study, no correlation was found between pre-operative platelet dysfunction and postoperative bleeding using PW (74). Thus far, no studies have been performed evaluating the performance of PW as a POC test in the (massive) bleeding patient.

Multiplate®. Multiple electrode platelet aggregometry, performed on whole blood after addition of agonists, produces a trace with results expressed as the area under the curve parameter in aggregation units. The essay is relatively simple to perform and will produce a result in under 10 min after sampling, except when ADP is used as an agonist, as this requires a pre-analytical 30-min equilibration period. As reference ranges may vary widely it is advised to obtain own reference ranges. Multiplate can be used to detect the effects of both medication and (cardiac) surgery on platelet

function (75,76). Platelet aggregation was found to be reduced during and after cardiopulmonary bypass, especially in patients who were transfused, suggesting that Multiplate might have a role in guiding platelet transfusion (77). However, the Multiplate assay has not been validated for low platelets count, making this device unsuitable in clinical decision making in the patient with massive blood loss.

• *Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT)*
Several POC tests measuring PT, INR and aPTT have been developed. They are mainly used because of their rapidity to obtain results in the ambulant setting and less in the setting of major surgery or to guide blood product replacement. Table 4 summarizes the devices that provide a rapid portable way to check PT, INR and aPTT. Although a variety of devices have been developed, the use of POC for measuring the PT (other than for monitoring the INR) is not widespread. Further, POC testing of INR is limited in situations of altered blood composition as extremes of haematocrit (<25% or >55%), haemolysis and increased viscosity all affect test outcome (48). Also, the combined use of warfarin and (low molecular weight-) heparin may impair the accuracy of POC testing for INR (78,79). Finally, only very few studies have been published on the use of POC PT/INR in a clinical setting (80). Despite adequate test validity, the wide variation in outcomes of POC testing for aPTT makes it less accurate compared to measurement of aPTT in the central laboratory (81). Furthermore, only few devices developed to monitor aPTT, are available for purchase. In the next section we will focus on the commercial available devices on PT and aPTT that have been evaluated in the bleeding patient.

CoaguChek systems. The Coaguchek XS (plus) is a POC test of INR, mainly used to monitor treatment with oral anticoagulants. Although the test looses its sensitivity with INR above 3.5, it is rapid and highly accurate compared with the reference test both in patients undergoing major surgery and in the intensive care setting (82-84). Moreover, in patients undergoing major surgery it could reduce the transfusion of fresh froze plasma, suggesting that the use of POC testing of PT could lead to a more rational use of blood products (85). However, INR is not an appropriate test in the analysis of massive blood loss because it is standardised for control on

vitamin K antagonists. As a consequence, results may be misleading in the context of coagulopathy due to dilution, consumption or liver disease.

Table 4. POC tests of secondary haemostasis: INR, PT, aPTT, ACT

POC test	Principle	Clinical Use	Limitations
PT/ INR: • Coaguheck (X)S plus • INRatio Monitoring System • Hemochron • Thrombotrak • Protime 3 • i-Stat • Gem PCL • Cascade POC	measurement of changes in electrochemical impedance	• monitoring treatment with oral VKA • assessment extrinsic pathway of coagulation	• extremes of haematocrit (<25% or >55%), haemolysis and increased viscosity affect test outcome • poor correlation with laboratory INR at values >3.5 • inaccurate measurements when warfarin is combined with LMWH • only limited data on PT/ INR measurements in clinical setting. • baseline PT/INR in patients with lupus anticoagulans before starting oral VKA
aPTT: • Hemochron Devices	measurement of changes in electrochemical impedance	• mainly used to monitor activated clotting time, but also PT, aPTT and ACT • assessment intrinsic pathway of coagulation	• outcome affected by type of activator, temperature and haematocrit. • unclear correlation with laboratory measured PT and aPTT, especially: - INR in VKA users - aPTT in Heparin users
ACT: • Hemochron • Hepcon HMS • i-STAT Modification of ACT: • High Dose Thrombin Time (HiTT)	automated heparin-protamin titration method	• monitoring ACT and high dose heparin • monitoring of heparin	• during CPB the ACT is variable for Hemochron • Hepcon:overestimation of heparin dose in CPB surgery & sensitive to haemodilution and hypothermia • poor correlation between HiTT and ACT

PT; prothrombin time, aPTT; activated partial thromboplastin time, INR; international normalized ratio; ACT; activated clotting time, VKA; vitamin K antagonist, LWMH; low molecular weight heparin, CPB; cardiopulmonary bypass

INRatio Monitoring System. This POC test of INR is mainly used as a self testing device for oral anticoagulation. It measures the change in impedance of the blood-reagent-mixture during the process of coagulation and determines PT which correlated well with the standard laboratory measured PT (86). No data exists on device performance in the analysis of coagulation in the bleeding patient.

Hemochron devices. The Hemochron is a POC electromechanical device, mainly used to monitor activated clotting time, but it is also marketed to monitor PT and aPTT. There are conflicting reports, concerning the correlation between Hemochron POC measured- and standard laboratory measured- PT and aPTT. During orthotopic liver transplantation, determination of PT and aPTT using the Hemochron device showed good correlation with laboratory coagulation assays (87). In contrast, a poor correlation was found between standard laboratory measurements for INR in patients using vitamin K antagonists and for aPTT in patients on intravenous heparin and in patients post CBP surgery (88), 89). The Hemochron technique has not been validated for its potential role in both the prediction and the treatment of massive blood loss.

• *Activated Clotting Time (ACT)*

The principle of this screening test of the intrinsic coagulation pathway is based on mechanical detection of coagulation, which is optically measured, after activation (with celite, kaolin or Cilicia parts) in a cartridge. ACT is mainly used to monitor high concentrations (>1 IU/ml) of unfractionated heparin in patients undergoing cardiopulmonary bypass (CPB). Although the ACT is considered the gold standard, the test correlates poor with heparin plasma levels. Further, no formal external quality assessment programme exists for the ACT; lyophilized quality control plasma samples, commercially available, should be used on a regular base to ensure that test results are within reference ranges (89). The type of activator used, hypothermia and haematocrit all can affect test outcome (90). Several systems for the estimation of free heparin have been developed; all are fundamentally based on the test principle of aPTT (table 4).

In contrast to control conditions, where a linear relationship was found between ACT and heparin, ACT was highly variable during CPB using the Hemchron device (91,92). In patients undergoing vascular surgery, both a poor and a good correlation were found between the Hemochron low range heparin ACT with respectively aPTT and anti-Xa activity (93,94). Using a heparin-protamin titration system, the Hemochron RxDx, led to a significant reduction in postoperative blood loss, as well as the amount of heparin and initial doses of protamin used during CPB (95). During CPB procedure and haemodialysis, i-STAT provided measurements of clotting time quite similar to Hemochron ACT, which were significantly correlated with heparin levels (96).

Given the conflicting reports, further studies are needed to determine whether maintaining heparin levels during CPB using a protocol based on ACT is more effective in preventing consumptive activation of the haemostatic system, reducing bleeding, and minimizing the use of blood products, when compared with conventional monitoring of heparin concentration. As ACT is only a global test of haemostasis and cannot, without modification, distinguish between the causes of coagulopathy, the technique has no role in both the prediction and the treatment of massive blood loss.

• *Thrombin Time (TT)*

The TT measures the conversion of fibrinogen to fibrin following the addition of exogenous thrombin and was historically used to monitor patients on unfractionated heparin. Because the classical TT becomes immeasurably prolonged at high concentrations of unfractionated heparin, both the High Dose Thrombin Time (HiTT, Hemchron) and the Hepcon HMS have been employed to overcome this problem. HiTT was found a useful assay for monitoring heparin effects during cardiac surgery, even during hypothermia and hemodilution, although HiTT correlated poor with ACT during CPB (97). The heparin concentrations determined with the Hepcon/HMS instrument did not agree with laboratory determination of heparin concentration during CPB (98). The TT, and its modifications, have no role in neither the prediction of bleeding nor in the analysis of the patient with massive bleeding.

• *Tests of overall coagulation; Thromboelastography.*

Thromboelastography measures the visco-elastic properties of a developing clot and provides real time information about the quality of the clot and aspects of formation and lysis. Moreover, the coagulation status is assessed at the patients temperature and in whole blood, taking into account the interaction with all other cellular elements. The two instruments currently available are: TEG® Haemostasis Analyzer and the ROTEM® (Table 5). The basic principle of thromboelastography involves incubation of whole blood in a heated sample cup into which a pin is suspended. The pin and the cup or the cup alone oscillates as the clot forms. The resulting thromboelastogram is a continuous graphic record of the physical shape of the clot during fibrin formation and subsequent lysis. There are minor mechanical differences between the two instruments, and the activators used differ with respect to potency. In the TEG® a pin is connected to a torsion wire, and clot formation generates a physical connection between the cup and pin that is converted to an electrical signal which is recorded via transducer and monitored via chart recorder. In the ROTEM® the pin is fixed on the tip of a rotating shaft, whereas the sample cup is stationary and the position of the axis is detected by reflection of light on a smaller mirror on the axis. Therefore, the results differ from each other and are not comparable between instruments (99-101).

Table 5. POC tests of global haemostasis; thromboelastography

POC test	Principle	Clinical use	Limitations
TEG® Haemostasis Analyzer ROTEM®	viscoelastic whole blood test modifications: - native or citrated samples - use of activators - use of heparinase	• detection of coagulopathy • guide to blood product and drug administration during cardiac- and hepatic surgery • management of massive haemorrhage • hypercoagulability screening	• outcome affected by type of activator, temperature and haematocrit. • methodology not standardized • significant intra- and inter-laboratory variability • no quantitative analysis of clotting or anticoagulant factors • correlation to classical coagulation tests is poor • no External Quality Assurance programme

Thromboelastography was initially used to guide transfusion in the setting of trauma-, cardiovascular-, and hepatic- surgery as the technique offers (the theoretical) advantage to provide insight into the underlying pathophysiology driving the complex and multifactorial haemostatic disturbances and to tailor haemostatic treatment (49,50,102-105). Since then, its use has been expanded to all other areas of haemostasis- and thrombosis- testing (106-110). Moreover, various guidelines recommend its possible role in both analyzing and managing haemostatic defects in patients with massive bleeding (9,111). However, studies evaluating the role of thromboelastography as a predictor of excessive bleeding after CPB show conflicting reports, with only minimal support in favor of its usefulness (112-114). Three prospective randomized trials (one blinded) demonstrated reduced transfusion requirements using an thromboelastography-guided transfusion algorithm, compared to a laboratory guided algorithm, in patients during complex cardiac surgery, whereas another study demonstrated no benefit (104,105,115,116). In hepatic surgery no prospective studies were performed on the preoperative thromboelstographic haemostatic profile and subsequent transfusion requirements. However, hyperfibrinolysis, complicating orthotopic liver transplantation, can preoperatively be predicted by thromboelastography (117). Also the treatment of fibrinolysis by aprotinin can readily be monitored by TEG (118). Although Kang et al advocated TEG-guided transfusion in hepatic surgery some 25 years ago, since then one randomized clinical trial has been performed demonstrating decreased transfusion of fresh frozen plasma, without effect on red blood cell transfusion or 3-year survival (50,119). However, the absence of guidelines and clear evidence as to the most appropriate threshold has left questions with regard to the clinical use of algorithm based replacement therapy in hepatic surgery (120). A recently published Cochrane review concluded that application of a TEG or ROTEM guided transfusion strategy seems to reduce the amount of bleeding but whether this has implications for the clinical condition of patients is still uncertain (121). They further concluded that there is an absence of evidence that TEG or ROTEM improves morbidity or mortality in patients with severe bleeding.

Even though there are several publications using thromboelastography it remains a research tool as the methodology is still not standardized which result into significant intra-and inter-laboratory variability as was recently demonstrated by the TEG-ROTEM Working Group (122). Moreover, it is important to realize that the technique does not provide any quantitative analysis of clotting or anticoagulant factors. Also the correlation to classical coagulation tests is poor. We demonstrated that the TEG variable "Maximum Amplitude" (corresponding to clot strength and related to platelet count) was insensitive in detecting the haemostatic effects of severe thrombocytopenia (< 50 x 10^9/L). Even more importantly, we found that platelet count not only affects the TEG parameter corresponding to clot strength, but also all other phases of plasmatic coagulation (123). As a consequence, the results of thromboelastography must be carefully interpreted correlating them to clinical conditions.

CONCLUSION

The classical coagulation tests for evaluation of bleeding disorders are especially useful in analyzing isolated defects in the coagulation cascade, whereas complex and multifactorial haemostatic disturbances, as seen in trauma and massive blood loss, are difficult to analyze. Despite it's shortcomings, it is important to realize that classical coagulation tests are well standardized, and that blood collection, sample handling and test running is performed by trained laboratory personal minimizing pre-analytical errors. Moreover, these tests are subject to strict internal- and external-quality control procedures making them reproducible and reliable. The investigation of haemostasis at the central laboratory versus POC testing have distinct objectives. The utility of the former for diagnosis and adjustment of therapeutics have been well demonstrated. In contrast, the experience with POC testing is relatively recent, and its utility for patient management remains to be demonstrated in prospective randomized clinical trials (124).

However, as several of the discussed POC test of haemostasis continue to evolve and have potential clinical benefit, it seems likely that these tests will be used on a larger scale in clinical practice. Therefore it is of importance that workers in the field of haemostasis remain aware of new developments and applications of these evolving techniques and assays. Furthermore, as these POC tests become increasingly used outside of the specialised laboratory, attention should be paid to validation, reliability and quality control. As far as POC platelet function is concerned, at this time no single POC test of platelet function has conclusively been shown to deliver reliable and reproducible results. POC tests that assess global haemostasis, e.g. thromboelastography, have potential for allowing a new look at the process of haemostasis. Many transfusion medicine specialists feel that POC assays and transfusion algorithms may provide an effective concurrent method in clinical decision-making and in the use of blood products in the surgical setting. Gradually, a shift of paradigm is seen in the management of massive blood loss, as two guidelines now propose near-patient testing, e.g. thromboelastography, if available, next to the assessment of classical coagulation tests, in characterising coagulopathy and guiding haemostatic therapy (9,111). However, there is no evidence that these assays give an advantage over conventional coagulations tests where there is access to rapid result reporting from the laboratory and in situations where the surgery is relatively low risk. (48,63). It is important to realize that the validity of POC tests of haemostasis has to be based on the achievement of accurate and reliable results leading to improved patient care. Still work needs to be done to standardise the methodology and its applications as well as to correlate clinical outcome with the measured parameters. Importantly, randomised studies evaluating thromboelastography-guided transfusion therapy versus fixed ratios of blood components on overall mortality in patients with massive blood loss are highly warranted.

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3

In Normal Controls, both Age and Gender affect Coagulability, as measured by Thrombelastography®

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Anesth Analg 2010;110:987-94.

ABSTRACT

BACKGROUND: Our objective was to analyze the effects of age, gender and the use of oral contraceptives (OCs) on coagulation using Thrombelastography® (TEG), a single test to analyze both plasma coagulation factors and cellular elements in whole blood.
METHODS: TEG variables were measured in native whole blood and in recalcified citrated blood from 120 healthy adults (60 men and 60 women) with various ages and in an additional 29 healthy women using OCs.
RESULTS: We observed hypercoagulability in females compared to males and in women using OCs compared with age-matched nonusers. Moreover, we found hypercoagulability with aging. Using the method of Bland Altman (Lancet 1986;1:307-10), we demonstrated no correlation between TEG measurements in native and recalcified citrated blood.
CONCLUSIONS: Aging, female gender, use of OCs and low-normal hematocrit levels have significant procoagulant effects. TEG measurements in native and recalcified citrated blood are not interchangeable, as indicated by differences between the 2 measurements ranging from 20% in maximum amplitude to 246% in clotting time. Furthermore, the limits of agreement strongly exceeded clinical acceptability to conclude interchangeability.

INTRODUCTION

Thrombelastography® (TEG) is classically used in situations where point-of-care testing of haemostasis is desired. There is also increasing clinical interest in assessment of the prothrombotic tendency by TEG, for example in thrombophilia screening, but also in prediction of arterial or venous thrombosis in the general population (1). An advantage of TEG over conventional tests of haemostasis is that it is performed on whole blood, taking into account the role of interacting blood elements such as phospholipid-bearing cells and platelets (2,3). The technique offers a rapid overview of the cumulative effect of all the individual components of haemostasis, without having to analyze each of these components separately. Further, TEG provides information about the quality of the clot as well as the dynamics of its formation and its lysis. The different parts of the TEG tracing correspond to specific deficiencies in coagulation factors or inhibitors of coagulation, use of anticoagulants, platelet count as well as platelet function and fibrinogen level. Depending on the shape of the TEG tracing, the haemostatic condition of a patient can be defined as normal, hypocoagulable or hypercoagulable. TEG is considered more sensitive than routine assays in detecting hypercoagulability (4-7). The interpretation of TEG data is, however, hampered by the lack of a validated large series of reference values, especially from elderly persons and from subjects with a physiologic hypercoagulable state like users of oral contraceptives (OCs) and pregnant women (8,9). The purpose of the present study was both to establish reference ranges and to asses whether age, gender and use of OCs influence haemostasis as measured by TEG in fresh native whole blood in healthy persons. Although TEG was originally designed as a bedside monitor for native whole blood, recalcified citrated blood is used as an alternative to perform tests within the laboratory. Therefore we also performed TEG in recalcified citrated native whole blood to explore whether both TEG techniques show comparable results and are thus exchangeable.

MATERIALS AND METHODS

The institutional review board approved the study, and informed consent was obtained from all study participants. Sixty healthy male and 60 healthy female subjects, age distributed equally between 19 and 87 years, participated in the study. The following exclusion criteria were used: coagulation disorders, use of anticoagulants, use of oral contraceptives (except for the substudy, see below), use of acetylsalicylic acid within the past 10 days, use of nonsteroidal anti-inflammatory drugs within the last 24 hours, renal diseases or plasma concentration of creatinine more than 120 μmol/L, and liver disease or increased plasma concentration of aspartate aminotransferase (>50 U/L) or alanine aminotransferase (>50 U/L). A history of thromboembolism was permitted. The effects of OCs on coagulation were examined by comparing TEG variables of 29 healthy young female OC users with age-matched controls.

Blood sampling and assays

Blood samples were obtained simultaneously for TEG analysis and standard laboratory and coagulation tests (i.e. complete blood count, white blood cell differentiation, creatinine, aspartate aminotransferase, alanine aminotransferase, prothrombin time [PT], activated partial thromboplastin time [aPTT], fibrinogen concentration and antithrombin [AT]). Venous blood samples were collected by vein puncture at the antecubital fossa, using a 19 gauge butterfly needle. One single experienced examiner obtained all blood samples. To minimize the effects of venous endothelial damage by using a tourniquet, the first aspirate of 10 mL blood was discarded. Blood was collected into a 20-mL polypropylene syringe to prevent contact activation of clotting by glass. Some of the collected blood, 3.5 mL, was filled into two Vacutainers (Greiner Bio-One, Kremsmünster, Austria), containing 0.5 mL coagulation sodium citrate 3.2% for subsequent standard coagulation tests and TEG analysis (recalcified citrated native whole blood; C). All standard coagulation tests were performed on the STA-R coagulation analyzer (Roche, Diagnostica Stago, Asnières, France): PT with Thromborel S reagents and aPTT with Actin FS reagents (Dade Behring, Marburg, Germany), fibrinogen with excess thrombin (BioPool, Umea, Sweden) according to the Clauss method, and AT with thrombin as enzyme (STAchrome ATIII, Roche kit).

Normal values for these variables in our laboratory are PT 11 to 16 sec, aPTT 26 to 36 sec, fibrinogen 1.7 to 3.5 g/L, AT 75% to125%.

Thrombelastographic assays were carried out using a computerized TEG coagulation analyzer (Model 5000; Haemoscope Corp., Niles, IL). All analyses were performed with TEG disposable cups and pins as devised by the manufacturer. Polypropylene and polyethylene pipettes were used to handle reagents and blood. TEG analyses were performed in native whole blood and recalcified citrated native whole blood. For TEG analysis in native blood, 360 µl whole blood was pipetted into the prewarmed TEG cup and measurements were performed within 6 minutes of sampling (10). Recalcification and TEG measurements in citrated blood were performed after storage at room temperature for 1 hour, as described previously[11]. Twenty microliters of 200-mM calcium chloride was pipetted into the prewarmed TEG cup. The citrated blood was gently inverted to ensure mixing of the sample. Next, 340 µl citrated blood was added to the TEG cup.

The following TEG variables were recorded: the reaction time (R-time, min), representing the rate of initial fibrin formation, the clotting time (K-time, min), representing the time until a fixed level of clot firmness is reached, the angle (α, degrees), which is closely related to K-time and represents the rate of clot growth, the maximum amplitude (MA, mm), is a measurement of maximum strength or stiffness of the developed clot, the shear elastic modulus strength (SEMS or G, dynes/cm^2) is a parametric measure of clot firmness expressed in metric units calculated from MA as follows:

$$G = (5000 \times MA) / (100 - MA).$$

Furthermore we calculated the coagulation index (CI), which is an overall measurement of coagulation, using the following equation:

$$CI = (-0.1227R + 0.0092K) + (0.1655MA - 0.0241\alpha) - 5.022.$$

R-time, K-time and α are prolonged by anticoagulants and factor deficiencies, but they can also be affected to a degree by platelet dysfunction or thrombocytopenia. MA is especially influenced by platelet count and platelet function as well as fibrinogen level.

In addition to “classical” TEG variables, we made velocity calculations, describing thrombus generation (TG) during blood clotting, from the signature graph produced by thrombelastography. These variables give additional information on the kinetics of the coagulation cascade as these variables represent the more parametric measurements of clot propagation (12). The following TG parameters were recorded: the Maximum TG (MTG, dynes/cm^2/s); this variable presents the first derivate of the velocity of the increase in clot strength, beginning as G begins to increase and ending after clot strength stabilizes. The information from this parameter is equivalent to the information from the alpha angle, however MTG provides a more parametric evaluation than the determination of α. The second variable is Time to Maximum rate of TG (TMG, seconds), which is the time it takes to reach MTG. Finally we determined Total TG (TTG, dynes/cm^2), which is the total positive area under the velocity curve, representing the total change in elastic resistance until clot strength stabilization occurs. MTG and TTG are expressed using metric units of elastic resistance that accurately describe changes in clot strength.

Statistical analysis

TEG values were presented as mean and standard deviation (SD). All normal values of TEG variables were calculated by the mean value ± 2 x SD. Group comparisons were made by the Student’s t test if normally distributed or Mann-Whitney U test if the distribution was skewed. Where appropriate, age was treated as a dichotomous variable, with a cutoff value of 50 years. Linear regression was used to quantify the associations of TEG parameters with age, sex and relevant blood test. In these analyses, Pearson correlation coefficients (r^2) were calculated, as well as the level of significance (with a null-hypothesis r = 0). Multivariable regression analyses were performed to obtain adjusted correlation coefficients of TEG variables with age, sex and blood tests. All variables univariately associated with the TEG variable of interest at a p level of <0.10 were included in the multivariable regression analysis. Co-linearity between independent variables was assessed prior to the multivariable analyses. To evaluate the level of agreement of TEG values in noncitrated whole blood versus citrated whole blood, the method of Bland Altman was used (13). The Bland-Altman plot is a tool for the presentation of method comparison between studies.

The range in limits of agreements determines how much the new method (citrated native whole blood) differs from the old one (native whole blood). All individually reported p values must be interpreted within the concept of an explorative testing, rather than formal hypothesis testing. Therefore, a correction for multiple testing was not applied. Analyses were performed using commercially available computer software (Statistical Analysis System, version 8.0, SAS Institute, Cary, NC, and SPSS 17.0 software for Windows, SPSS, Chicago, IL).

RESULTS

We studied 120 healthy adults, 10 per age decade, mean age 50 ± 17 years, 60 women and 60 men. Another 29 healthy female controls using OC, of which more than 60% used a second generation OC, were analyzed. In Table 1 the demographics of all subgroups are shown, with a comparison of male with female (non-OC users) and a comparison of OC users with age-matched non-OC users. Apart from hemoglobin and creatinine levels, there were no statistically significant differences between the male and female control group.

Table 1. Healthy control demographics.

Variable	Male (n=60)	*p*	Female (n=60)	*p*	Female OC+ (n=29)
Age (yr)	50 ± 18	ns	49 ± 18	<0.001	26 ± 6
Leukocytes, x 10^9/L	7.1 ± 1.2	ns	7.3 ± 1.5	ns	7.4 ± 1.4
Hemoglobin, mmol/L	9.0 ± 0.5	<0.001	8.1 ± 0.6	ns	7.8 ± 0.6
Platelets, x 10^9/L	243 ± 54	ns	257 ± 49	0.01	275 ± 54
Creatinine, µmol/L	98 ± 18	<0.001	85 ± 16	ns	80 ± 7

Values are mean ± SD. OC+, using oral contraceptives; ns, not significant.

Effect of gender on TEG parameters

Mean values ± SD of TEG variables, measured in native and citrated whole blood, and classical coagulation tests in the 3 subgroups are presented in Table 2. There were statistically significant differences in coagulability between male and female subjects. Except for R-time measured in citrated blood (p=0.06), all other TEG variables in the female group were statistically significant in hypercoagulability when compared with the male group, whereas no statistically significant differences were found between the classical coagulation tests in both sexes. In females we observed a significantly faster and higher initial rate of fibrin formation as illustrated by a shorter R- and K-time. The female group also had a significantly higher rate of clot growth, represented by a wider α angle. Moreover, MA, SEMS, and CI were significantly higher in the female group. The latter suggests that both maximum clot strength as well as the viscoelastic property of the formed clot were higher in women compared with men. Also, the novel TG variables were different in men compared with women. According to these variables, thrombin was generated significantly quicker (TMG) and with greater velocity (MTG) in women. Finally, the value for TTG was also significantly higher in women.

Effect of OCs on TEG Variables

We compared TEG variables of the group using OCs with an age-matched subgroup of women who did not take OCs (n = 22). In our study women using OCs had significant higher values respectively for MA, SEMS, and TTG generated. Apart form a higher fibrinogen level in women using OCs, no other statistically significant differences were found in classical coagulation tests between groups (Table 2).

Effect of age on TEG Variables

Weak to moderate correlations between age and most TEG variables were observed in both sexes. The strongest correlations emerged between age and MA (r = 0.47), SEMS (r = 0.47) and TTG (r = 0.47). The scatter diagrams and regression equations of these 3 TEG variables versus age are presented in Figure 1, a-c. Observing the different TEG variables per decade, a gradual change toward hypercoagulability with increasing age can be seen. Significant differences in TEG variables were found in

both sexes when subjects younger than 50 years were compared with subjects older than 50 years (Table 3). In the classical coagulation tests, the older male group also had statistically significant shorter aPTT (p=0.03), higher fibrinogen levels (p=0.003) and lower AT percentages (p<0.001) compared to the younger male group. Females above 50 years had significant higher fibrinogen levels (p<0.001) and demonstrated a trend towards a shorter aPTT (p=0.055) compared to the younger female group.

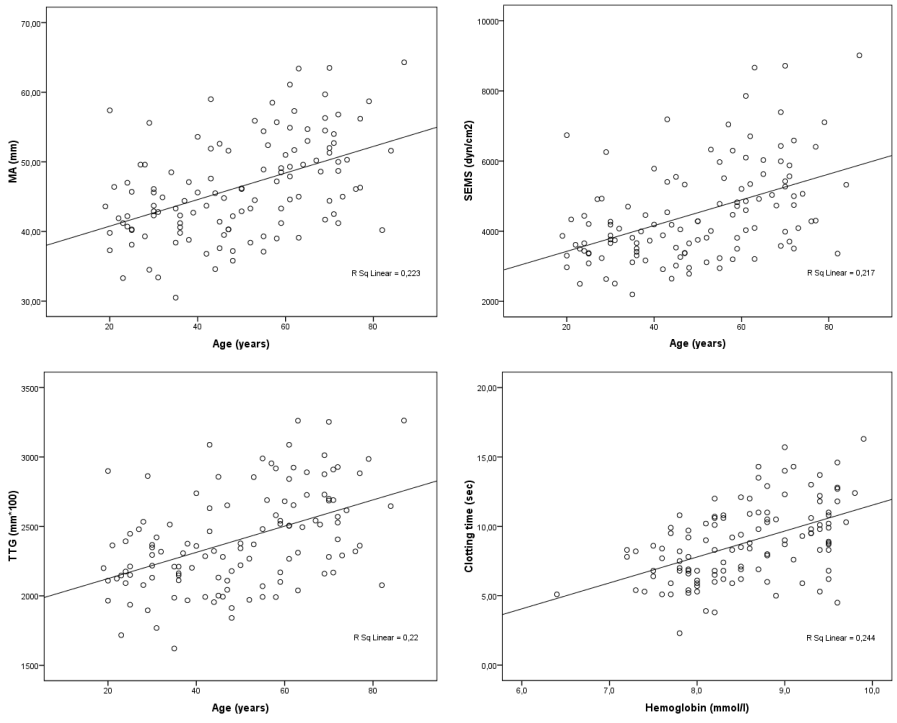

Figure 1a - d.

Correlation and linear regression of age with maximal clot strength (maximum amplitude), maximal clot elasticity (shear elastic modulus strength), and the total amount of thrombus that is generated (total thrombus generation) and of hemoglobin with the time to reach a fixed level of clot firmness (clotting time); R Sq linear = correlation coefficient.

Table 2. Thrombelastography variables and classical coagulation tests in male and female subgroups, in Native and Citrated whole blood.

Variable	Male (n=60)	p	Female (n=60)	Female OC+ (n=29)	p	Female OC- (n=22)
R time						
N	23.1 ± 5.7	< 0.001	19.9 ± 4.5	20.9 ± 4.5	ns	20.0 ± 4.4
C	11.2 ± 2.2	0.06	10.5 ± 1.8	10.8 ± 2.4	ns	10.5 ± 1.6
K time						
N	10.0 ± 2.7	< 0.001	7.7 ± 2.5	7.9 ± 2.2	ns	8.4 ± 2.3
C	2.9 ± 0.9	< 0.001	2.3 ± 0.5	2.4 ± 0.6	ns	2.4 ± 0.5
A						
N	22.4 ± 6.0	< 0.001	28.5 ± 8.2	25.7 ± 9.4	ns	26.2 ± 7.4
C	53.1 ± 8.2	< 0.001	59.0 ± 5.2	54.2 ± 10.3	ns	57.4 ± 5.2
MA						
N	44.0 ± 6.6	< 0.001	48.7 ± 6.9	49.9 ± 6.2	0.003	45.1 ± 4.4
C	56.5 ± 6.2	0.005	59.3 ± 4.2	61.8 ± 5.9	0.007	57.7 ± 3.8
SEMS						
N	4066 ± 1209	< 0.001	4934 ± 1432	5140 ± 1323	0.004	4172 ± 822
C	6728 ± 1715	0.01	7424 ± 1285	8501 ± 2957	0.02	6913 ± 1074
CI						
N	-3.75 ± 1.83	< 0.001	-2.39 ± 1.58	-2.36 ± 1.43	ns	-2.93 ± 1.35
C	0.37 ± 1.14	0.008	0.85 ± 0.76	1.28 ± 1.17	0.03	0.64 ± 0.72
MTG						
N	3.6 ± 1.0	< 0.001	4.5 ± 1.4	4.4 ± 1.6	ns	4.2 ± 1.2
C	9.7 ± 2.9	< 0.001	11.5 ± 2.3	11.6 ± 3.1	ns	10.6 ± 2.0
TMG						
N	1927 ± 873	< 0.001	1573 ± 391	1626 ± 493	ns	1588 ± 412
C	791 ± 161	0.04	737 ± 129	767 ± 160	ns	729 ± 113
TTG						
N	732 ± 104	< 0.001	804 ± 113	823 ± 103	0.004	745 ± 69
C	940 ± 101	0.02	978 ± 70	1020 ± 97	0.005	951 ± 62
PT	12 ± 0.6	ns	13 ± 0.6	13 ± 0.6	ns	13 ± 0.6
aPTT	31 ± 3	ns	31 ± 3	30 ± 3	ns	32 ± 3
Fibrin.	3.0 ± 0.5	ns	3.0 ± 0.5	3.3 ± 0.6	<0.005	2.7 ± 0.5
AT	100 ± 13	ns	103 ± 10	104 ± 10	ns	104 ± 10

Values are mean ± SD. ns; not significant; OC+, using oral contraceptives; OC-, not using oral contraceptives; R-time (min), reaction time; K-time (min), clotting time; A, alpha angle (degree); MA (mm), maximum amplitude; SEMS (dynes/cm^2), shear elastic modulus strength; CI, coagulation index; MTG (dynes/cm^2/sec), maximum thrombus generation; TMG (sec), time to maximum thrombus generation; TTG (dynes/cm^2), total thrombus generation; N; native whole blood, C: citrated whole blood. PT (sec), prothrombin time; aPTT (sec), activated partial thromboplastin time; Fibrin. (g/L), fibrinogen; AT (%), antithrombin.

Table 3. Thrombelastography variables and classical coagulation tests for both sexes, comparing subjects younger than 50 years with age older than 50 years.

Variable	Male < 50 (n=30)	p	Male > 50 (n=30)	Female < 50 (n=30)	p	Female > 50 (n=30)
R time						
N	24.4 ± 4.6	ns	21.7 ± 6.4	20.5 ± 4.2	ns	19.4 ± 4.8
C	11.8 ± 2.0	0.02	10.5 ± 2.3	10.7 ± 1.5	ns	10.3 ± 1.95
K time						
N	10.9 ± 2.6	0.007	9.1 ± 2.4	8.4 ± 2.6	0.04	7.14 ± 2.3
C	3.2 ± 0.9	0.006	2.6 ± 0.7	2.5 ± 0.5	0.001	2.1 ± 0.4
A						
N	20.4 ± 4.5	0.009	24.4 ± 6.7	26.1 ± 7.8	0.02	31.0 ± 8.2
C	50.0 ± 7.9	0.002	56.3 ± 7.3	57.0 ± 5.0	0.003	60.9 ± 4.8
MA						
N	41.4 ± 5.5	< 0.001	47.0 ± 6.3	45.0 ± 5.7	<0.001	52.0 ± 6.3
C	53.7 ± 6.1	< 0.001	59.3 ± 5.0	57.8 ± 3.9	0.005	60.8 ± 4.0
SEMS						
N	3665 ± 876	0.002	4583 ± 1299	4253 ± 1064	<0.001	5635 ± 1419
C	5999 ± 1520	< 0.001	7482 ± 1603	6950 ± 1120	0.004	7883 ± 1285
CI						
N	-4.5 ± 1.5	< 0.001	-3.0 ± 1.8	-3.0 ± 1.4	0.002	-1.8 ± 1.5
C	-0.18 ± 1.01	< 0.001	0.93 ± 1.0	-0.6 ± 0.69	<0.001	1.1 ± 0.77
MTG						
N	3.3 ± 0.6	0.02	3.9 ± 1.2	4.2 ± 1.2	ns	4.8 ± 1.6
C	8.7 ± 2.5	0.006	10.7 ± 2.9	10.6 ± 2.1	0.002	12.5 ± 2.3
TMG						
N	2104 ± 1085	ns	1744 ± 540	1612 ± 385	ns	1545 ± 411
C	841 ± 146	0.02	741 ± 161	754 ± 122	ns	721 ± 136
TTG						
N	686 ± 84	< 0.001	780 ± 101	747 ± 94	<0.001	860 ± 102
C	898 ± 103	< 0.001	984 ± 79	953 ± 64	0.005	1002 ± 67
PT	12.3 ± 0.7	ns	12.5 ± 0.5	12.7 ± 0.6	ns	12.3 ± 0.5
aPTT	31.8 ± 3.0	0.03	30.3 ± 2.3	31.5 ± 2.7	ns	29.8 ± 3.8
Fibrin.	2.8 ± 0.5	0.003	3.2 ± 0.5	2.8 ± 0.4	<0.001	3.2 ± 0.5
AT	106.7 ± 10.8	<0.001	93.3 ± 12.5	104.4 ± 9.1	ns	102.2 ± 11

Values are mean ± SD. R-time (min), reaction time; K-time (min), clotting time; A (degrees), alpha angle; MA (mm), maximum amplitude; SEMS (dynes/cm^2), shear elastic modulus strength; CI, coagulation index; MTG (dynes/cm^2/sec), maximum thrombus generation; TMG (sec), time to maximum thrombus generation; TTG (dynes/cm^2), total thrombus generation; PT (sec), prothrombin time; aPTT (sec), activated partial thromboplastin time; Fibrin. (g/L), fibrinogen; AT (%), antithrombin.

Association of other variables with TEG Variables

In addition to the relationship of TEG variables with age and gender, we also studied the association among TEG variables, PT, aPTT, and blood cell counts. In a univariate analysis TEG variables were associated with gender, age, hemoglobin level, platelet count, aPTT and fibrinogen level (data not shown). Remarkably, no correlation was found between the PT and any of the TEG variables. In multivariable analysis, age remained statistically correlated with the MA and with the derived variables SEMS and TTG (Table 4). Furthermore, gender was associated with all TEG variables except TMG. Regarding classical coagulation tests, the lack of correlation between platelet count and TEG variables is surprising but in the context of a platelet count in the normal range. However, aPTT showed a correlation with the initial fibrin formation as demonstrated by a positive correlation with R-time, K-time and the time to MTG and a negative correlation with the α angle. Fibrinogen was associated with all TEG variables, except the R-time.

Finally, a positive correlation between hemoglobin level and the clotting time (K-time; $r = 0.24$) was present. With increasing hemoglobin level, a prolongation of the time to reach a fixed degree of viscoelasticity, as a result of fibrin buildup and cross-linking, was observed (Figure 1d). Hemoglobin level had comparable effects on TEG variables in both sexes.

TEG Variables in fresh whole blood versus recalcified citrated whole blood

We determined limits of agreement for the differences in native and recalcified citrated whole blood for each of the TEG variables (Table 5 and Figure 2). Our data show a lack of correlation between the TEG measurements in both types of blood samples, due to the very large limits of agreement. As is shown in Figure 2, for the R-time and K-time the lack of agreement increased with an increase of the R-time and K time. For MA, this was less pronounced. The mean difference between the K-time measured in native blood and recalcified citrated whole blood was 246% (Table 5).

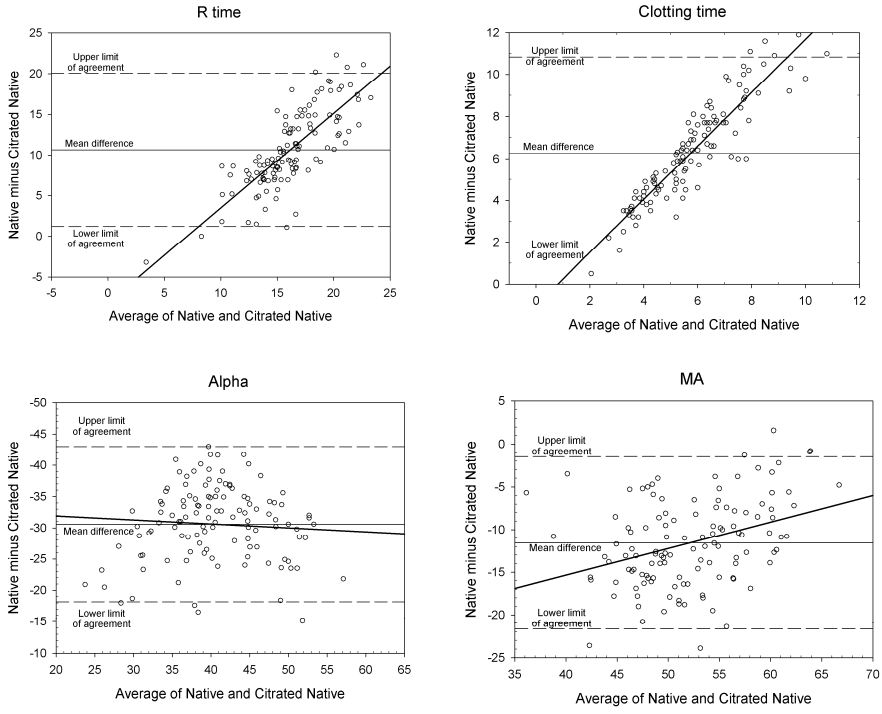

Figure 2.

Scatter plots for the time until initial fibrin formation occurs (R-time), the time until a fixed level of clot firmness is reached (clotting time), the rate of clot growth (alpha), and the maximal clot strength (maximum amplitude) of mean versus difference of the fresh and recalcified citrated whole blood thromboelastography values.

Table 4. Significant correlation coefficients by multivariate analysis among variables age, gender, classical coagulation tests and TEG variables in Native whole blood.

	R-time	K-time	A	MA	SEMS	CI	MTG	TMG	TTG
Age	ns	ns	ns	0.32	0.32	0.19	ns	ns	0.30
Gender	-0.26	-0.22	0.27	0.24	0.22	0.3	0.23	ns	0.24
Hb	ns	0.24	ns	ns	ns	ns	ns	ns	ns
Platelet	ns	ns	ns	ns	ns	ns	ns	ns	ns
aPTT	0.36	0.24	-0.22	ns	ns	-0.21	ns	0.21	ns
Fibrinogen	ns	-0.27	0.28	0.37	0.37	0.25	0.27	ns	0.40

ns, not significant; R-time, reaction time; K-time, clotting time; α, alpha angle; MA, maximum amplitude; SEMS, shear elastic modulus strength; CI, coagulation index; MTG, maximum thrombus generation; TMG, time to maximum thrombus generation; TTG, total thrombus generation; Hb, hemoglobin; aPTT, activated partial thromboplastin time

Table 5. Limits of agreement for differences in Native and Citrated whole blood.

	Mean difference	Mean diff in % change in native compared to CN	95% CI of mean difference	Limits of agreement
R time (min)	10.6	101%	9.8 to 11.5	1.2 to 20.1
K time (min)	6.3	246%	5.8 to 6.7	1.7 to 10.8
A (degree)	-30.6	-55%	-31.7 to -29.4	-43.0 to -18.2
MA (mm)	-11.6	-20%	-12.5 to -10.6	-21.6 to -1.5
SEMS (dynes/cm^2)	-2576	-36%	-2788 to -2364	-4873 to -280
CI	-3.7	-243%	-3.9 to -3.4	-6.4 to -0.9
MTG (dynes/cm^2/sec)	-6.7	-61%	-7.2 to -6.2	-11.3 to -2.1
TMG (sec)	983	132%	862 to 1105	-330 to 2297
TTG (dynes/cm^2)	-192	-20%	-206 to -177	-365 to -18

R-time, reaction time; K-time, clotting time; A, alpha angle; MA, maximum amplitude; SEMS, shear elastic modulus strength; CI, coagulation index; MTG, maximum thrombus generation; TMG, time to maximum thrombus generation; TTG, total thrombus generation; CN, citrated native

DISCUSSION

In the present study we assessed reference values for TEG variables in a large population of healthy volunteers. Our report is unique because we are the first who study a population with a well-balanced age and gender distribution. Moreover, we describe both classical and dynamic (TG) TEG variables and compare these with classical coagulation tests. Finally, we performed TEG measurements in both native and recalcified citrated whole blood in order to see whether both techniques produce the same results and are thus interchangeable.

In our study population we demonstrated significant gender differences in coagulation, with a more procoagulant TEG profile in women compared to men. In women, not only a faster rate of fibrin formation was observed (clotting time 7.7 ± 2.5 min [women] vs. 10.0 ± 2.7 min [men]; $p<0.001$), but also a greater ultimate clot strength with better viscoelastic properties (MA 48.7 ± 6.9 mm [women] vs. 44.0 ± 6.6 mm [men]. In contrast, there were no gender differences using classical coagulation tests in these same subjects. The observed gender-related differences in coagulation remained with aging and, as a consequence, could not solely be explained by the effects of female sex hormones. Neither could pregnancy nor the use of OCs be held responsible for this difference, because both conditions were excluded from this sub-analysis. Lang and colleagues found a trend towards enhanced coagulation in women compared with men (14). However, in their female reference population, use of OCs was not an exclusion criteria which may have affected the outcome.

Although the exact mechanism is unknown, OC use increases the risk of venous thrombosis (8). From this study, we conclude that women using OC have higher ultimate clot strength with better viscoelastic properties compared to age-matched controls not using OC. In contrast, Zahn and co-workers, using celite as activator, found no significant TEG changes in women using low estrogen dose OCs (15). Adding coagulation activators such as celite results into shortening of the initial part of the TEG line, making rapid interpretation of the coagulation process possible. However, this same activation may be at the cost of subtle information from this initial part

of the TEG line. Therefore, we would like to emphasize on the use of nonactivated whole blood in TEG when studying circumstances in which coagulation differences are considered to be small.

Gorton et al. have reported significant gender-related differences in TEG variables, with a significant procoagulant trend from men through nonpregnant women to pregnant women (16). However, these results may have been biased, since half of the non-pregnant women were taking third generation OC and only patients with an average age of 30 years were included. In our study we could demonstrate that the effect of gender is not limited to the younger age group but is a phenomenon that persists with aging.

Aging is associated with hypercoagulability and considered as an important risk factor for venous thrombosis (17). We demonstrated a tendency towards hypercoagulability in both sexes with advancing age. Although there was no statistically significant difference per decade, there was a trend that may have been relevant with greater numbers of healthy study subjects. Moreover, statistically significant differences in most TEG variables were found comparing the age group younger than 50 years with the group older than 50 years, as shown in Table 3. TEG variables mostly influenced by age were MA, SEMS, and TTG, all linked with the building up and cross-linking of fibrin, leading to a stronger thrombus with better viscoelastic properties. For the classical coagulation tests, aging was accompanied by a shortening in aPTT in the male subgroup and a rise in fibrinogen level in both genders. Others demonstrated that in patients undergoing orthopedic surgery, aging was associated with procoagulable TEG variables (18). However, in contrast to our study, age was not equally distributed in that study because patients older than 80 years were overrepresented. More recently no age-related differences in kaolin-activated TEG variables in healthy children could be identified and no significant differences between children and adults were observed (19). A possible explanation, given by the investigators, is that the process of activation of the blood samples could have affected the degree of age-related haemostatic differences.

TEG with recalcified citrated blood is used as an alternative to noncitrated blood in situations in which immediate TEG determination is not practically feasible. The use of a citrated blood sample, however, generates different results to that seen when a native sample is used (11,20). Others demonstrated that coagulation analyses using blood that has been exposed to citrate and recalcified afterwards, do not yield reliable depictions of the natural dynamics of the blood coagulation process (21). We studied TEG variables in noncitrated as well as in recalcified blood and demonstrated an important lack of agreement between the 2 techniques. As a consequence we consider both techniques not exchangeable. Both gender- and age-related differences in TEG variables were more prominent when measured in noncitrated than in citrated whole blood. The latter suggests that preparation of the blood sample (native versus recalcified citrated native) is important for the detection of subtle age- and gender-related hypercoagulable states.

We demonstrated by multivariate analysis a strong reversed correlation between hemoglobin level (still within the normal range) and the speed to reach a certain level of clot strength (= K or clotting time). In other words, a more rapid fibrin buildup was observed in control persons with lower hematocrit levels. The importance of erythrocytes in haemostasis has been described before. In the absence of platelets, erythrocytes can contribute to thrombin generation through exposure of pro-coagulant phospholipids at their outer cell membrane (22). Iselin and co-workers demonstrated that a progressive isolated reduction in hematocrit from 40% to 10% resulted in an accelerated blood coagulation profile resulting in increased clot strength as measured in celite-activated citrated blood (23). Others found a state of relative hypercoagulability (shortened R-time) immediately after a rapid 10% loss in circulating blood volume (24). Because we examined healthy persons with their hematocrit within the normal range, we can only speculate on an optimal hematocrit favoring haemostasis.

There were a number of limitations to this study. First, we did not correct for multiple testing. Although such an adjustment of p-values, e.g., by Bonferroni or Hochberg step-up Bonferroni, can be applied to our data, we a priori decided not to apply such

an adjustment because the purpose of all hypothesis testing was not to identify a single significant result, but rather to evaluate differences between groups on all the TEG variables as a whole. Second, although operation procedures were strictly followed in accordance with the Thrombelastograph Operation Manual, the (native) TEG values in general were hypocoagulable compared to what has been historically reported by several laboratories worldwide. As TEG outcome is influenced by many variables (blood collection site, sample stability, repeat sampling, and also modifications of the technique like citration or adding activators) we think that for correct interpretation of TEG results each center should perform analyses in a standardized way, with assessment of outcomes against (own) normal ranges derived from samples handled in the same manner. Finally, in clinical practice, where TEG is used as a rapid point of care test of haemostasis, coagulation activators are often added to the blood samples. As we used only whole blood samples without activators, the study observations may not be applicable to other methods of TEG measurement.

In summary, we present reference values in a large random population, equally distributed for age and gender. We observed significant procoagulant effects of aging, female gender, use of OC and low-normal hematocrit levels on TEG variables. We also demonstrated an important lack of agreement between TEG measurements in native and recalcified citrated blood and considered both techniques not exchangeable. Our study underlines the sensitivity of TEG over classical coagulation tests in detecting these subtle, probably physiological, differences in haemostasis.

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4

Thrombocytopenia affects Plasmatic Coagulation as measured by Thrombelastography®

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Blood Coagulation and Fibrinolysis 2010, 21:389-397

ABSTRACT

BACKGROUND: Thrombelastography® (TEG) is used as a point-of-care test of haemostasis. Different components of the test tracing are considered to reflect various parts of the haemostatic system and to distinguish low platelet count, platelet dysfunction or both from lack of plasmatic coagulation factors. To analyze the influence of one single element of the coagulation system, namely the platelet count, we used TEG serially in patients with well documented transient thrombocytopenia.
STUDY DESIGN AND METHODS: A total of 189 TEG analyses were performed from 16 patients with a hematological malignancy in remission, receiving consolidation courses of chemotherapy. TEG outcome using unmanipulated and citrated blood samples at a median of 11 times (range 1-17) in the same patients during the decrease of platelet count in response to chemotherapy were compared with outcomes in 120 healthy adults from various age categories.
RESULTS: We found a correlation (r = 0.7; $p<0.001$) between TEG clot strength (maximum amplitude) and platelet count. Moreover, platelet count was correlated with respectively the initial rate of clot formation (reaction time and clotting time), the rate of clot growth (alpha angle), and also with maximum thrombus generation, time to maximum thrombus generation and total thrombus generation.
CONCLUSION: We conclude that platelet count not only affects the strength of clot formation, as was expected, but also all other phases of plasmatic coagulation. Citration of the blood sample, aiming at easy storage of the material, masked some of the important biological parameters of coagulation.

INTRODUCTION

To determine the relative contribution of the different components of haemostasis, conventional coagulation tests like prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen concentration and platelet count are measured. The limitation of these classical coagulation assays is that only information on the plasma coagulation factor levels is provided. Moreover, as most of these tests are performed in a central laboratory it often takes time to obtain results, making the test less suitable as a perioperative test of haemostasis. In recent years the Thrombelastograph® (TEG; Haemoscope Corporation, Niles, Illinois, USA) has gained popularity as a useful point-of-care coagulation monitor in the perioperative setting (1-4). TEG is a whole blood test that gives information not only on plasma coagulation factors but also on the influence of platelets, leukocytes and erythrocytes on haemostasis, defining TEG as a more physiological instrument (5). The different components of the TEG profile reflect different parts of the coagulation process and are used to assess the need for blood component therapy (6).

The purpose of the present study was to examine the influence of one single component of the coagulation system, the platelet count, on all available TEG parameters as measured in both native and citrated native whole blood. Although often used in clinical practice, we decided not to add activators of the coagulation process, in order to improve interpretation of the initial part of the TEG line. We investigated patients with a hematological malignancy receiving chemotherapy, and assessed TEG multiple times during the chemotherapy-induced decrease of platelet count. Thus, in a single patient all variables remained constant except for the changing platelet counts. The analyses were performed in a standardized way and results were compared to ranges obtained from healthy controls. We hypothesized that the platelet count correlates with the strength of clot formation, and, that this TEG parameter is sensitive in detecting clinically relevant thrombocytopenia.

MATERIALS AND METHODS

Patients and controls

The institutional review board approved the study and informed consent was obtained from all patients (n=16) and control persons (n=120). Patients were treated for hematological malignancies and admitted for their second or third course of chemotherapy. For all patients the underlying disease was in complete remission after the preceding chemotherapy, precluding any effect of the underlying disease on the coagulation process. Also the bone marrow function had to be restored to normal with concomitant normal peripheral blood counts. In these 16 patients we studied the TEG performance multiple times (median 11, range 1-17) during the decrease and subsequent recovery of platelet count. The 120 controls were healthy adults from all age categories. For both study patients and controls, the following exclusion criteria were used: coagulation disorders, use of anticoagulants, use of acetylsalicylic acid within the preceding 10 days, use of non steroidal anti-inflammatory drugs within the last 24 hour, renal diseases or plasma concentration of creatinin more than 120 μmol/L and liver disease or increased plasma concentration of aspartate aminotransferase (ASAT) (> 50 U/L) or alanine aminotransferase (ALAT) (> 50 U/L). Patients with septicemia, diffuse intravascular coagulation or thrombosis during the study period were also excluded. Finally patients with a recent history (<1 week) of platelet or red blood cell (RBC) transfusion were also not eligible.

Blood sampling and assays

Blood samples were obtained simultaneously for TEG analysis and standard laboratory and coagulation tests [i.e. complete blood count, white blood cell differentiation, creatinine, ASAT, ALAT, PT, aPTT, fibrinogen and anti-thrombin (AT)]. Platelet aggregation assays with ADP, adrenalin, ristocetin and arachidonic acid were performed once at study entrance to exclude an underlying thrombopathic disorder. Venous blood samples were drawn from an indwelling central venous catheter (patients) or collected by vein puncture at the antecubital fossa using a 21-gauge butterfly needle (controls). Two examiners obtained all blood samples, both experienced in performing phlebotomy. Blood was collected into a 20-mL

polypropylene syringe to prevent contact activation by glass. To minimize the effects of using a tourniquet and to exclude effects of heparin in the central venous catheter, the first aspirate of 10 mL blood was discarded. In addition to the native whole blood (native) test, some of the collected blood, 3.5 mL, was filled into two Vacutainers (Greiner Bio-One, Kremsmünster, Austria), containing 0.5 mL Coagulation Sodium Citrate 3.2% for subsequent standard coagulation tests and TEG analysis (recalcified citrated native whole blood; citrated native). All standard coagulation tests were performed on the STA-R coagulation analyzer (Roche, Basel, Switzerland); PTT with Thromborel S reagents and aPTT with Actin FS reagents (DadeBehring, Marburg, GmbH, Marburg, Germany), fibrinogen with excess thrombin (BioPool US Inc., Ventura, California, USA) according to the Clauss method and AT with thrombin as enzyme (STAchrome ATIII, Roche kit). Normal values for these parameters are PT 11-16 s, aPTT 26-36 s, fibrinogen 1.7-3.5 g/L and AT 75-125%.
Thromboelastographic assays were carried out using a computerized TEG coagulation analyzer (Model 5000; Haemoscope Corp). All analyses were performed with TEG disposable cups and pins as devised by the manufacturer. Polypropylene and polyethylene pipettes were used to handle reagents and blood.

TEG measurements (and standard blood laboratory evaluation) took place at study entrance, before starting chemotherapy, and 3 times a week afterwards until the platelet count dropped below 100 x 10^9/L. From that point on, TEG analyses and blood tests were performed daily until a patient needed a RBC transfusion (hemoglobin < 8 g/dl) or a platelet transfusion (platelet count < 10 x 10^9/L).

For TEG analysis in native blood, 360 mL whole blood was pipetted into the prewarmed TEG cup and measurements were performed within 6 minutes from sampling (7). Recalcification and TEG measurements in citrated native blood were performed after storage at room temperature (RT) for 1 hour, as described previously (8). Twenty mL 0.2 mol/L calcium chloride was pipetted into the prewarmed TEG cup. The citrated native blood was gently inverted to ensure mixing of the sample. Then 340 mL citrated native blood (RT) was added to the prewarmed TEG cup. The following TEG parameters were recorded: the reaction time (R, min), representing

the rate of initial fibrin formation; the clotting time (K, min), representing the time until a fixed level of clot firmness is reached; the angle (α, degrees), which is closely related to K-time and represents the rate of clot growth; the maximum amplitude (MA, mm), is a measurement of maximum strength or stiffness of the developed clot; the shear elastic modulus strength (SEMS or G, dynes/cm^2) is a parametric measure of clot firmness expressed in metric units calculated from MA as follows:

$$G = (5000 \times MA) / (100 - MA).$$

R time, K time and α are prolonged by anticoagulants and coagulation factor deficiencies, maximum amplitude is especially influenced by platelet count and platelet function as well as fibrinogen level.

In addition to the former "classical" TEG parameters we made velocity calculations, describing thrombus generation during blood clotting, from the signature graph produced by TEG. These dynamic parameters are considered to give better insight into the initial part of the TEG trace by differentiating the entire initial time course (9). The following Thrombus generation parameters were recorded: Maximum Thrombus Generation (MTG), Time to Maximum rate of thrombus Generation (TMG) and Total Thrombus Generation (TTG). First, MTG (dynes/cm^2/s), representing the velocity by which clot strength increases, beginning as SEMS starts to increase and ending after clot strength has been stabilized. The information from this parameter is equivalent to the information from the alpha angle, however MTG provides a more parametric evaluation than the determination of α. The second variable is TMG (s), which is the time it takes to reach MTG. Finally we determined TTG (dynes/cm^2), which is the area under the velocity curve, representing the total change in elastic resistance until clot strength stabilization occurs. MTG and TTG are expressed using metric units of elastic resistance that accurately describe changes in clot strength. A characteristic velocity profile of whole blood clot formation is shown in Figure 1.

Statistical analysis

For statistical analysis SPSS 14.0 software for Windows (SPSS Inc., Chicago, Illinois, USA) was used. Variables are expressed as mean ± SD. Mean values of the TEG parameters were tested by an unpaired t-test comparing controls with study patients

with different levels of thrombocytopenia. Significance levels were set at 0.05 (two-tailed). The correlations of changes in platelet level and individual TEG variables were calculated using Spearman rank correlations.

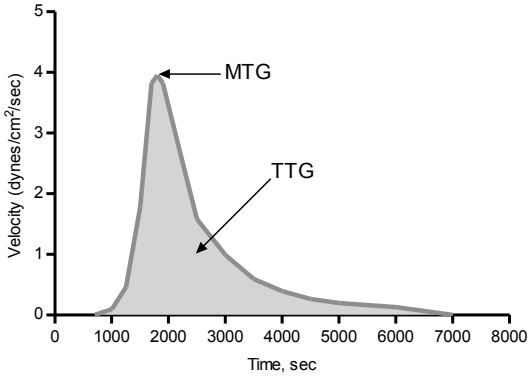

Figure 1.

Characteristic velocity profile of whole blood clot formation with maximum thrombus generation, which represents the velocity by which clot strength increases, beginning as SEMS starts to increase and ending after clot strength has been stabilized. TTG is the area under the velocity curve, representing the total change in elastic resistance until clot strength stabilization occurs. MTG, maximum thrombus generation; TTG, total thrombus generation.

RESULTS

To obtain our own reference values, 120 controls, age 50 ± 18 years, range 20-80 years, 10 women and 10 men per decade, were studied. In Table 1 the values of the TEG parameters for native blood and citrated native blood are presented. The study group consisted of 16 patients (seven women and nine men), median age 45 years, range 18-62 years. Ten patients were treated for acute leukemia, five for multiple myeloma and one for Hodgkin's lymphoma. In these patients a total of 189 TEG analyses were performed. In 16 patients, 121 TEG analyses in native blood were performed (median 7, range 1-15). In 10 patients 68 TEG analyses in citrated native blood were performed (median 7, range 2-17). At study entrance each patient had normal classical coagulation tests as well as normal platelet aggregation assays (data not shown).

Table 1. TEG parameters in controls and in patients at different platelet counts, in Native (N) and Citrated Native (CN) whole blood.

			Patients Platelet count x10^9/L			
TEG parameter		**Controls**	**>100**	**100-50**	**50-25**	**<25**
	N	(n=120)	(n=46)	(n=21)	(n=13)	(n=9)
	CN	(n=120)	(n=36)	(n=12)	(n=10)	(n=9)
R-time (min)	N	22 ± 5	26 ± 13*	46 ± 32*	52 ± 27*	68 ± 25*
	CN	11 ± 2	13 ± 6	10 ± 3	17 ± 12*	15 ± 3*
K-time (min)	N	9 ± 1	9 ± 4	19 ± 11*	30 ± 13*	46 ± 11*
	CN	3 ± 1	3 ± 2	3 ± 1	7 ± 3*	9 ± 3*
α (°)	N	26 ± 8	30 ± 10	17 ± 9*	10 ± 6*	5 ± 1*
	CN	56 ± 7	54 ± 12	51 ± 7*	34 ± 10*	28 ± 7*
MA (mm)	N	46 ± 7	58 ± 9	54 ± 10	45 ± 10	30 ± 5*
	CN	58 ± 6	61 ± 17	55 ± 10	49 ± 9*	37 ± 6*
SEMS (dyne/cm^2)	N	4506 ± 1392	7288 ± 3371	6323 ± 2471	4333 ±1654	2182 ± 489*
	CN	7082 ± 1545	8504 ± 3103	6608 ± 2464	5046 ±1543*	3049 ± 727*
MTG	N	4,1 ±1,3	3,2 ± 1,3*	2 ± 1,1*	1,6 ± 0,9*	1,4 ± 0,6*
(dynes/cm^2/sec)	CN	10,6 ± 2,8	6,6 ± 3,2*	5,6 ± 2,7*	2,6 ± 1,4*	2,8 ± 1,5*
TMG (sec)	N	1747 ± 693	981 ± 809*	771 ± 242	1747 ± 1956*	1184 ± 653*
	CN	764 ±147	2340 ± 1404*	3639 ± 2621*	4359 ± 2572*	5088 ± 4254*
TTG (dynes/cm^2)	N	769 ± 114	559 ± 117*	524 ± 83*	426 ± 167*	400 ± 63*
	CN	959 ± 88	552 ± 91*	511 ± 83*	449 ± 85*	347 ± 44*
TMA (min)	N	49 ± 10	56 ± 18*	99 ± 45*	117 ± 40*	140 ± 30*
	CN	31 ± 4	34 ± 10	34 ± 5	51 ± 16*	55 ± 14*
R+K (min)	N	30 ± 8	35 ± 13	66 ± 32*	82 ± 27*	107 ± 25*
	CN	13 ± 3	16 ± 6	14 ± 4	24 ± 12*	24 ± 3*

*p<0.05 different between control and study group. R-time, reaction time; K-time, clotting time; α, alpha angle; MA, maximum amplitude; SEMS, shear elastic modulus strength; MTG, maximum thrombus generation; TMG, time to maximum thrombus generation; TTG, total thrombus generation; TMA, time to maximum amplitude; R+K, R-time plus K-time.

Native whole blood

Thrombocytopenia strongly influenced the TEG performance. Of the 121 TEG analyses, 30 (25%) could not be evaluated because no fibrin formation was detected. This phenomenon was related to decreasing platelet counts. No coagulation at all could be detected in 18 out of 29 (62%) patients with platelet counts less than 25 x 10^9/L, in five out of 18 (28%) patients with platelet counts between 25-50 x 10^9/L and in seven out of 74 (9%) patients with platelet counts more than 50 x 10^9/L. In all of these cases, however, classical coagulation tests showed normal plasmatic coagulation as measured by PT, aPTT, AT and fibrinogen (data not shown).

Relation between platelet count and TEG parameters

Fibrin formation could be measured in 91 patient samples by TEG. Figures 2 and 3 illustrate the effect of decreasing platelet count on TEG tracings, respectively velocity profiles, as measured in one patient. Identical TEG patterns were found in all other study patients with increasing thrombocytopenia. Table 1 shows the results of TEG parameters from controls and patients with decreasing platelet counts. Bar charts presenting TEG parameters found at different platelet counts are shown in Figure 4 and 5. At a platelet count below 100 x 10^9/L, we found an increase in time to clot initiation (R-time and K-time were significantly prolonged) as well as a decrease in clot propagation (α-angle) compared to controls. Remarkably, only at platelet levels below 25 x 10^9/L, clot strength (maximum amplitude) became significantly inferior compared to the control group. Clot propagation (MTG) and total change in elastic resistance (TTG) declined significantly compared to controls even at platelet count more than 100 x 10^9/L (in this group one-third of the patients had a platelet count below 150 x 10^9/L). Statistically significant correlations were found between platelet count and respectively maximum amplitude ($r = 0.7$; $p<0.0001$), R-time ($r = -0.5$; $p<0.0001$) K-time ($r = -0.7$; $p<0.0001$), a-angle ($r = 0.8$; $p<0.0001$), MTG ($r = 0.6$; $p<0.0001$), TMG ($r = -0.3$; $p<0.008$) and TTG ($r = 0.6$; $p<0.0001$) (Figure 6a-g). In other words, both the time until clot initiation and propagation increased, and the ultimate clot strength and (change in) viscoelastic properties decreased significantly at lower platelet counts. In contrast, no significant correlations were found between platelet count and PT ($r = -0.2$, $p=0.1$), aPTT ($r = -0.08$, $p=0.5$) and fibrinogen levels ($r = -0.2$, $p=0.1$).

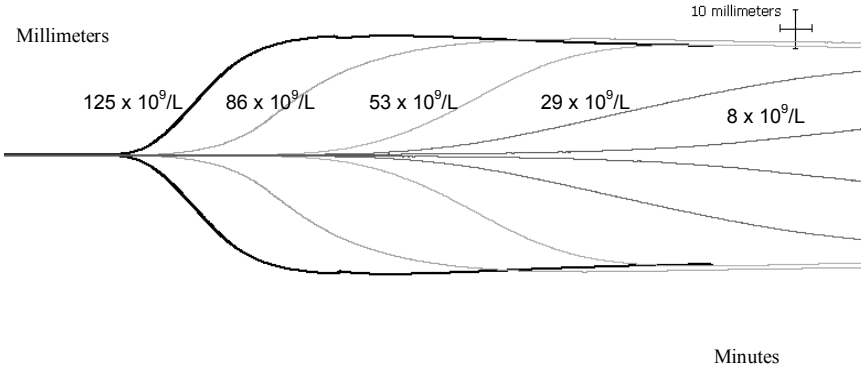

Figure 2.

Classical thrombelastography tracings in the same patient at decreasing platelet counts.

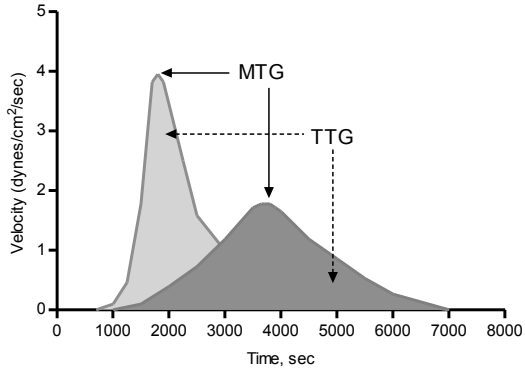

Figure 3.

Novel thrombelastography velocity profiles in the same patient at normal platelet counts (light grey area) and at low platelet count (dark grey panel). MTG, maximum thrombus generation; TTG, total thrombus generation.

Predictive value of TEG parameters regarding low platelet count

As TEG maximum amplitude is considered the parameter most reflecting functional thrombocytopenia, we calculated the sensitivity of maximum amplitude in detecting clinically significant thrombocytopenia (< 50 x 10^9/L). We found a sensitivity of 79%, with a specificity of 93% and a positive and negative predictive value of respectively 79% and 94% of TEG maximum amplitude in detecting important thrombocytopenia. The test characteristics for all TEG parameters are shown in Table 2.

Table 2. Predictive value of TEG parameters regarding low platelet count (<50x10^9/L), in Native (N) and Citrated Native (CN) whole blood.

TEG parameter		Test characteristic			
		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
R-time	N	35	100	100	73
	CN	65	92	77	87
K-time	N	96	67	50	98
	CN	30	72	30	72
A	N	96	76	58	98
	CN	85	80	63	93
MA	N	79	93	79	94
	CN	85	82	65	95
MTG	N	100	13	28	100
	CN	100	8	29	80
TMG	N	5	97	33	86
	CN	5	96	33	73
TTG	N	100	3	25	100
	CN	100	0	28	100

PPV = positive predictive value, NPV = negative predictive value.
R-time, reaction time; K-time, clotting time; A, alpha angle; MA, maximum amplitude; SEMS, shear elastic modulus strength; MTG, maximum thrombus generation; TMG, time to maximum thrombus generation; TTG, total thrombus generation.

Thrombelastography and bedside monitoring

TEG is considered a "near-patient" bedside test allowing rapid assessment of haemostasis. A fast way to obtain an early impression of the TEG curve is to look at the initial part of the TEG trace represented by time to maximum amplitude (TMA) and the sum of R- and K- time. The results of TMA and R+K time at different platelet counts, compared to controls are shown at the bottom of Table 1. This demonstrates that with platelet counts less than 100 x 10^9/L it took about 1 - 1.5 hours to get this "early" impression, whereas with platelet counts of less than 25 x 10^9/L it took 1.5 - 2.5 hours to obtain results.

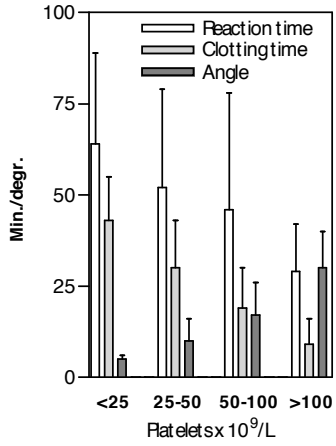

Figure 4.

Bar charts representing reaction time (R-time), clotting time (K-time) and α-angle in patients at different platelet counts in native whole blood. Parameters are presented as mean ± SD.

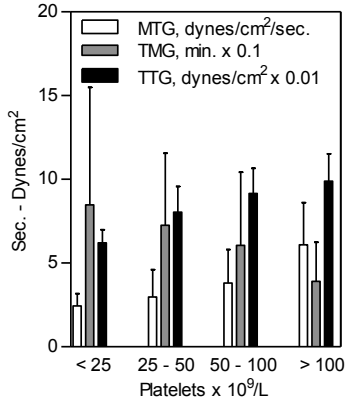

Figure 5.

Bar charts representing maximum thrombus generation, time to maximum thrombus generation and total thrombus generation in patients at different platelet counts in native whole blood. Parameters are presented as mean ± SD. MTG, maximum thrombus generation; TMG, time to maximum thrombus generation; TTG, total thrombus generation.

Recalcified citrated native whole (citrated native) blood.

All of the 68 analyses in citrated native blood could be evaluated because fibrin formation was detected (R-time, K-time or both were detectable).

Relation between platelet count and TEG parameters

Table 1 shows the effect of decreasing platelet count on TEG parameters. We found a correlation between platelet count and clot strength (maximum amplitude; r = 0.75; p=<0.001). At platelet counts below 50 x 10^9/L, maximum amplitude became significantly smaller than the maximum amplitude in healthy controls. No significant correlation was found between platelet count and clot initiation (R-time; r = 0.2; p=0.07). Significant correlations were found between platelet count and respectively K-time (r = -0.3; p=0.03), a-angle (r =;0.6; p<0.0001), maximum amplitude (r = 0.7; p<0.0001), SEMS (r = 0.7; p<0.0001), MTG (r = 0.4; p=0.003), and TTG (r = 0.6; p<0.0001). In other words, in citrated native blood, platelet count influenced both (the velocity of) clot propagation as well as the ultimate strength and elasticity of the formed clot.

Predictive value of TEG parameters regarding low platelet count

With citrated native blood, the sensitivity of a maximum amplitude in detecting clinically significant thrombocytopenia (< 50 x 10^9/L) was 85%, with a specificity of the test of 82% and a positive and negative predictive value of respectively 65% and 95%. The test characteristics for all TEG parameters are shown in Table 2.

Thromboelastography and bedside monitoring

The results of TMA and R+K time at different platelet counts, compared to controls, are shown at the bottom of Table 1. After storage for 1 hour (equilibration time), it took another 0.5-1 hour to obtain an early impression of the coagulation profile at platelet counts below 25 x 10^9/L.

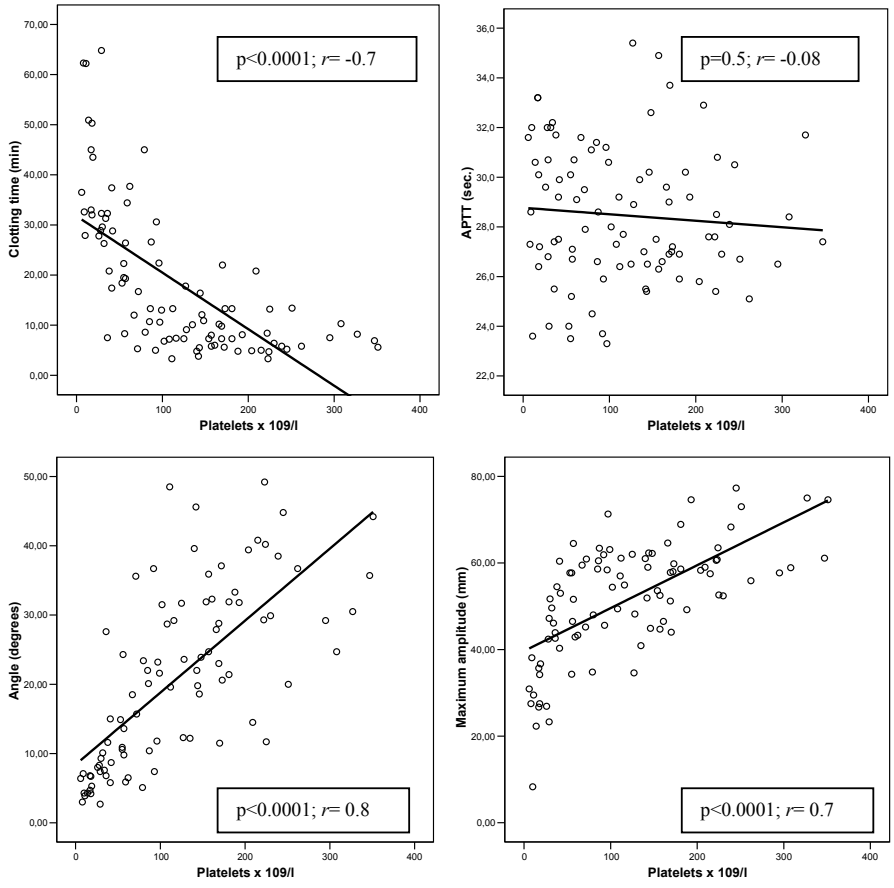
Clotting time (min)
p<0.0001; r= -0.7
Platelets x 109/l
APTT (sec.)
p=0.5; r= -0.08
Platelets x 109/l
Angle (degrees)
p<0.0001; r= 0.8
Platelets x 109/l
Maximum amplitude (mm)
p<0.0001; r= 0.7
Platelets x 109/l

Figure 6a-d.

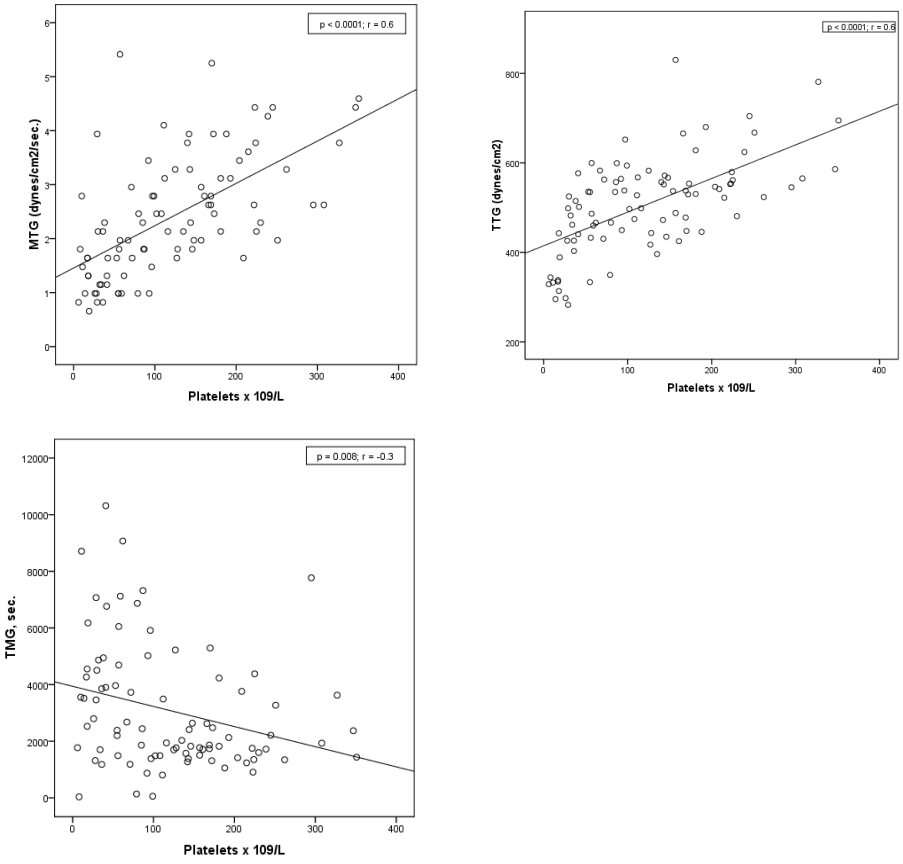

Figure 6e-g.

Correlation of platelet count and, respectively, clotting time (K-time), activated partial thromboplastin time, α-angle, maximum amplitude, maximum thrombus generation, total thrombus generation and time to maximum thrombus generation in patients in native whole blood. aPTT, activated partial thromboplastin time; MTG, maximum thrombus generation; r, Spearman rank correlation coefficient; TMG, time to maximum thrombus generation; TTG, total thrombus generation.

DISCUSSION

By studying the effect of different levels of thrombocytopenia on TEG parameters, we found that platelet count not only influenced the strength of clot formation (maximum amplitude) - an expected result - but also all other elements of the system. The finding of a correlation between platelet count (at levels < 100 x 10^9/L) and R- respectively K-time as well as α-angle and MTG, suggests an important role of platelets in initiating and propagating the coagulation cascade. A possible explanation for this observation is that platelets, but also platelet-derived micro particles, have a major role in providing membrane surface for the assembly of the reactants of the blood coagulation cascade (10). Moreover, platelets are a storage compartment for many proteins involved in blood coagulation and its regulation (11).

Low platelet counts (< 50 x 10^9/L) are considered a major risk factor for bleeding complications (12). TEG maximum amplitude is considered as the parameter best reflecting the effect of platelet count. However, in our study a significant decrease in maximum amplitude was noticed only after the platelet count dropped below 25 x 10^9/L. On the other hand, in native whole blood, both K-time and α-angle had a high sensitivity as well as high negative predictive values in detecting platelet counts below 50 x 10^9/L. This emphasizes again the importance of platelets in initiating the coagulation process. Also the novel thrombus generation parameters MTG and TTG were very sensitive and had high negative predictive values for low platelet counts, as measured in both native and citrated native whole blood. TEG is considered a bedside test, capable to deliver an impression of platelet count and function, coagulation proteins and fibrinolytic system within 30 minutes. We demonstrated that with decreasing platelet count both TMA and R+K time substantially prolong to more than 60 minutes, making rapid assessment of the coagulation process impossible. In order to circumvent this problem, perioperative TEG is performed with coagulation activator (e.g. kaolin, celite and diluted tissue factor). The coagulation activator makes blood clotting less dependent on platelets with R and K times more reproducible compared to R and K times obtained with non-activated samples. However, manipulation of the blood sample (by adding activators) is something

we wanted to minimize, in order to eliminate artificial influences and to get more realistic data. The present study suggests an important role for platelets in the entire coagulation process; compressing the initial part of the TEG trace may result in the loss of information on the initial phase of this cascade.

Another advantage of TEG over classical coagulation tests is that the assay provides information on the kinetics of the coagulation cascade as well as the quality and firmness of the resultant clot. The latter is calculated from the maximum amplitude and is defined as SEMS (dyne/cm^2). As with maximum amplitude, a significant reduction in SEMS was noticed compared to controls in native blood, only if the platelet count dropped below 25 x 10^9/L. In citrated native blood SEMS decreased earlier at platelet counts between 50 and 25 x 10^9/L.

TEG analysis was performed with both native and recalcified citrated native blood to determine which test technique gives best insight into the effects of thrombocytopenia on haemostasis. From a scientific point of view, TEG analysis with native blood is superior to TEG with citrated native blood and is for a long time considered the gold standard (13). However, in clinical practice TEG with citrated native blood seems more feasible because this can take place in the coagulation laboratory after one-hour equilibration time, compared to TEG with native blood, which has to take place within 6 minutes after sampling (14). The correlations found between TEG variables and platelet counts with both techniques are comparable. However, in case of severe thrombocytopenia (platelets <50 x 10^9/L), TEG performed with native blood showed a flat line in over more than 40% of the analyses, which makes it insensitive to detect these low platelet counts. Moreover, at these low platelet counts it often took more than two hours until TMA was reached, making the test with (nonactivated) native whole blood unsuitable as a rapid test of haemostasis. In contrast, TEG performed with citrated native blood at low platelet counts showed clot formation in all cases and TMA was reached within one hour in most of these cases. The latter suggests that citration could not completely prevent the activation of coagulation and platelets. This is in accordance with the findings of Camenzind et al, who demonstrated progressive acceleration of blood coagulation during 30

to 60 min citrate storage, with a decrease in reaction time, coagulation time and alpha angle (8). Others demonstrated that coagulation analyses using blood exposed to citrate followed by recalcification do not yield reliably depictions of the natural dynamics of blood coagulation processes (15).

Although TEG performances with different platelet counts were studied before (1,16,17), we consider our study unique at several points. First, multiple serial TEG measurements were performed in patients in whom only one component of the haemostatic system was altered, namely the platelet count. The latter is in contrast to studies in which the examined patients suffered from multiple haemostatic derangements, making analysis of the different underlying coagulopathies much more complex. Second, the fact that all measurements were performed ex vivo instead of using an in vitro model, makes our results more robust (18,19). More recently Larsen and co-workers, using ROTEM technology, found that the coagulopathy associated with thrombocytopenia in recalcified citrated blood, was characterized by a reduced MTG and maximum amplitude (20). Similar to our results they found that the minimum amount of platelets required to obtain a healthy MTG of whole blood clot formation was estimated to be 65 x 10^9/L. In their model, however, platelet preactivation cannot be excluded as they used a model of mixing platelet- poor plasma with platelet-rich plasma to obtain different levels of thrombocytopenia.
Third, we compared TEG outcomes in study patients with our own normal values obtained from a large series of healthy controls from all age categories. In all these controls TEG was performed in a uniform way by the same examiners, thereby reducing interobserver bias.
A limitation of our study is that we cannot preclude any direct or late effect of chemotherapy on the coagulation system. Also the underlying hematological condition, although in remission, may have affected study results. On the other hand, no significant changes were seen in the classical coagulation tests during the entire study period in these subjects.
Analysis of haemostasis by TEG cannot replace conventional coagulation tests, but can be seen as a valuable tool, giving extra insight into the kinetics of haemostasis, taking into account the interactions of all other cellular blood elements.

We conclude that platelet count not only affects the strength of clot formation, but also all other phases of plasmatic coagulation. Remarkably, TEG variable maximum amplitude was insensitive in detecting the haemostatic effects of severe thrombocytopenia (< 50 x 10^9/L).

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5

Transfused stored platelets have the same haemostatic function as circulating native platelets

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Vox Sanguinis 2010;99:123-130

ABSTRACT

BACKGROUND AND OBJECTIVES: As thrombelastography® (TEG) measures haemostasis in whole blood, we used this instrument to study whether transfused platelets (PLTs) have the same haemostatic function compared to native circulating platelets. Further we studied the effect of storage time on the haemostatic potential of platelet concentrates (PCs).

MATERIALS AND METHODS: During the decrease in PLT count after chemotherapy, TEG parameters were measured serially until the transfusion trigger was reached in 92 patients. TEG parameters for different ranges of native circulating PLT could be assessed, which were compared to ranges obtained in the thrombocytopenic period in which the patient received PLT transfusions. Finally we compared the haemostatic potential of fresh PCs (1-3 days) with PCs with longer storage time (4-5 days).

RESULTS: No differences could be found in haemostatic potential between native PLTs and transfused stored PLTs (all p values ≥0.1). The transfusion of fresh PLTs demonstrated better haemostatic effects then longer stored PLTs, measured 1h after transfusion. Both the time until a fixed level of clot firmness was reached (K-time) as well as the rate of clot growth (alpha angle) were superior for fresh PCs.

CONCLUSION: TEG is able to monitor the haemostatic effects of PLT transfusion, with comparable haemostatic properties of native circulating and transfused stored PLTs. Further, our data suggest that limited storage time is associated with a better haemostatic capacity. However, before TEG can be applied as a qualitative test in PLT transfusion, further research is needed with focus on clinical outcomes like bleeding episodes.

INTRODUCTION

Low platelet (PLT) counts (<50 x 10^9/L) are considered a major risk factor for bleeding complications (1). PLT transfusion is used therapeutically in patients with quantitative or qualitative PLT disorders who are actively bleeding, or as prophylaxis in patients who are at serious risk of bleeding (2). The main use of PLT transfusion is in the prevention of bleeding in patients with hematological malignancies who have bone marrow failure (3). Optimally, transfused PLTs should have the same functional properties as native circulating PLTs. The therapeutic effect of transfused PLTs however, cannot be predicted with certainty as efficacy is influenced by variables like storage and recipient's condition. The storage time of Platelet concentrates (PCs) is normally restricted to 5-7 days due to an increased risk of bacterial contamination. (4,5). Evaluation of the function of stored PLTs is only possible by the use of laborious and complicated in vitro tests and markers (6). Moreover, these in vitro tests do not provide information on the in vivo haemostatic potential of PLTs. In clinical practice PLT recovery is measured 1 and 24 hour after transfusion and corrected count increments (CCIs) are calculated in order to evaluate transfusion efficacy. In addition, the bleeding time used to be the most frequently used test of PLT function. However, the results of this test are considered poorly reproducible with poor sensitivity and specificity (7). Given the limitation of the bleeding time, several other tests in diagnosing and managing disorders of primary haemostasis and PLT function were developed (8). Thrombelastography® (TEG) is a whole blood test that gives information not only on plasma coagulation factors but also on the influence of PLTs, leukocytes and erythrocytes on haemostasis, defining it as a more physiological instrument to study haemostasis (9,10). The technique is classically used as a point-of-care test providing a global assessment of haemostasis in a graphic format. Different components of the test tracing are considered to reflect various parts of the haemostatic system and to distinguish low PLT count and/or PLT dysfunction from lack of plasmatic coagulation factors (11). The specificity of TEG was greater than that of bleeding time and PLT count in discriminating patients likely to benefit from transfusion of blood components during cardiac surgery (12). The Maximum Amplitude (MA), of the TEG curve, representing clot firmness, is seen as the parameter best reflecting the effect of PLT count (13-15).

We hypothesized that TEG might be a useful tool to improve the understanding and monitoring of the haemostatic quality and efficacy of PLT transfusion. The aim of the current study was to evaluate whether transfused PLTs have the same haemostatic function compared to native circulating PLTs, as measured by TEG. Moreover, we studied the effects of storage time on the haemostatic potential of PLTs.

MATERIALS AND METHODS

Study patients

The institutional review board approved the study and informed consent was obtained from all patients. Patients were treated for hematological malignancies and admitted for their second or third course of chemotherapy. For all patients the underlying disease was in complete remission after the preceding chemotherapy, precluding any effect of the underlying disease on the coagulation process. Also the bone marrow function had to be restored to normal with concomitant normal peripheral blood counts. For study patients the following exclusion criteria were used: coagulation disorders, use of anticoagulants, use of acetylsalicylic acid within the preceding 10 days, use of non steroidal anti-inflammatory drugs within the last 24 hours, renal diseases or plasma concentration of creatinine more than 120 µmol/L and liver disease or increased plasma concentration of aspartate aminotransferase (ASAT) (>50 U/L) or alanine aminotransferase (ALAT) (>50 U/L). Study patients who became febrile or demonstrated signs of soluble immune response suppressor or even sepsis, according to the ACCP criteria, were excluded from the study. Moreover, included patients had to have normal values for antithrombin (AT) and fibrinogen, making low grade disseminated intravascular coagulation (DIC, defined as systemic activation of pathways leading to and regulating coagulation, which result in the generation of fibrin clots that may cause organ failure with concomitant consumption of platelets and coagulation factors that may result in clinical bleeding) or consumptive coagulopathy very unlikely. Finally, patients who developed thrombosis, as demonstrated by ultrasonography, were also excluded from the study. In order to obtain our own normal (reference) values, we performed TEG and classical coagulation tests in 120 healthy adults; 60 women and 60 men, ten per

decade, age distributed equally between 19 and 87 years, mean age 50 ± 17 years. We used exactly the same exclusion criteria as used for our study population.

Study protocol

During the subsequent decrease in PLT count after chemotherapy, TEG parameters were measured serially until the transfusion trigger was reached (PLT count <10 x 10^9/L). Consequently, TEG parameters for different ranges of native circulating PLTs could be assed. We compared TEG parameters obtained before the start of the first PLT transfusion with those obtained in the thrombocytopenic period in which the patient was transfused with PCs (Figure 1). We assumed that in this aplastic period only transfused PLTs were circulating. Further we studied the effects on haemostasis comparing PCs with limited storage time (storage time 1-3 days) with PCs with longer storage time (4-5 days).

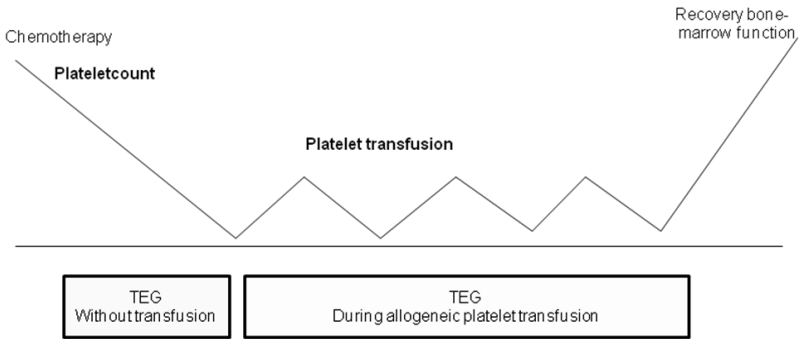

Figure 1.

Graphical presentation of study protocol

Blood sampling and assays

Blood samples were obtained simultaneously for TEG analysis and standard laboratory and coagulation tests [i.e. complete blood count, white blood cell differentiation, creatinine, ASAT, ALAT, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen and antithrombin (AT)]. Venous blood samples were drawn from an indwelling central venous catheter (patients) or collected by vein puncture at the antecubital fossa using a 21-gauge butterfly needle. Two examiners obtained all blood

samples, both experienced in performing phlebotomy. Blood was collected into a 20-mL polypropylene syringe to prevent contact activation by glass. To minimize the effects of using a tourniquet and to exclude effects of heparin in the central venous catheter, the first aspirate of 10 mL blood was discarded. All standard coagulation tests were performed on the STA-R coagulation analyzer (Roche, Basel, Switzerland); PT with Thromborel S reagents and aPTT with Actin FS reagents (Dade Behring, Marburg GmbH, Marburg, Germany), fibrinogen with excess thrombin (BioPool US inc, Ventura, CA, USA) according to the Clauss method and AT with thrombin as enzyme (STAchrome ATIII, Roche kit). Normal values for these parameters are PT 11-16 s, aPTT 26-36 s, fibrinogen 1.7-3.5 g/L and AT 75-125%.

Thrombelastographic assays were carried out using a computerized TEG coagulation analyzer (Model 5000; Haemoscope Corp., Niles, IL, USA). All analyses were performed with TEG disposable cups and pins as devised by the manufacturer. Polypropylene and polyethylene pipettes were used to handle reagents and blood.

TEG measurements (and standard blood laboratory evaluation) took place at study entrance, before starting chemotherapy, and 3 times a week afterwards until the PLT count dropped below 100 x 10^9/L. From that point on, TEG analyses and blood tests were performed daily until a patient needed a PLT transfusion (trigger was a PLT count <10 x 10^9/L).

For TEG analysis 360 mL whole blood was pipetted into the prewarmed TEG cup and measurements were performed within 6 minutes from sampling (16). No activators of coagulation were added to the samples. The following TEG parameters were recorded (between parenthesis normal values expressed as mean ± SD obtained in our laboratory in 120 normal persons): the reaction time (R, 22 ± 5 min), representing the rate of initial fibrin formation; the clotting time (K, 9 ± 3 min), representing the time until a fixed level of clot firmness is reached; the angle (α, 26 ± 8 degrees), representing clot growth and correlated to thrombin generation which results into interaction between platelets and fibrin; the maximum amplitude (MA, 46 ± 7 mm), is a measurement of maximum strength or stiffness of the developed clot; the

shear elastic modulus strength (SEMS or G, 4506 ± 1392 dynes/cm^2) is a parametric measure of clot firmness expressed in metric units calculated from MA as follows:

G = (5000 x MA) / (100 - MA).

R-time, K-time and α are prolonged by anticoagulants and coagulation factor deficiencies, MA is especially influenced by PLT count and PLT function as well as fibrinogen level.

Platelet transfusion

PCs were prepared from five buffy coats and one unit of plasma as described elsewhere (17). The platelet pooling system used was Optipure PLT Baxter (Utrecht, The Netherlands). At the time of issue of the PCs, the swirling effect had to be present and the bacterial screening test (BacT/Alert; Biomerieux, Marcy L'Etoile, France) had to be negative. Patients received PCs that had been stored between 1 and 5 days after blood collection according to standard Dutch guidelines (18). The corrected count increment (CCI) was calculated by subtracting the pre transfusion PLT (x 10^9/L) from the post transfusion PLT count (x 10^9/L), dividing this by the number of PLTs transfused (x 10^{11}) and multiplying this with body surface of the patient (m^2).

Statistical analysis

For statistical analysis SPSS 17.0 software for Windows was used. Variables are expressed as mean ± SD. Mean values for TEG parameters before and after transfusion with PCs were tested by a paired t-test. Comparisons between mean TEG values of native circulating PLTs with transfused allogeneic PLTs and between mean TEG values of "fresh" and "old" PCs, were made using an unpaired t-test. Significance levels were set at 0.05 (two-tailed).

RESULTS

Effect of PLT transfusion on haemostasis

In 92 patients (mean age 46 ± 15 yr, 42 women, 50 men) we studied the effect of PLT transfusion on haemostasis, as measured by both TEG and classical coagulation tests (Table 1). Overall we observed a good PLT recovery, as calculated by a 1 h CCI of 18 ±

7 and a 24 h CCI of 12 ± 8. No significant effects on classical coagulation tests could be observed after PLT transfusion.

Table 1. Classical coagulation tests and TEG parameters, measured before PLT transfusion, 1 h after PLT transfusion and 24 h after PLT transfusion.

	Before n=92	***p***	**After 1h n=91**	***p***	**After 24 h n=83**
Platelet	11 ± 5	<0.001	37 ± 13	<0.001	27 ± 14
CCI	-	-	18 ± 7	-	12 ± 8
PT	14.4 ± 4.6	ns	14.2 ± 2.8	ns	14.2 ± 2.9
aPTT	30.3 ± 7.3	ns	29.6 ± 5.4	ns	30.2 ± 6.0
AT	95 ± 16	ns	95 ± 15	ns	97 ± 14
Fibr.	5.5 ± 1.9	ns	5.4 ± 1.8	ns	5.7 ± 1.9
R	156 ± 131 (n=51)	<0.001	73 ± 60 (n=76)	ns	103 ± 86 (n=51)
K	43 ± 17 (n=13)	0.04	32 ± 20 (n=63)	0.03	39 ± 22 (n=36)
Alpha	2.2 ± 2.8 (n=51)	<0.001	10.0 ± 9.2 (n=75)	0.020	7.3 ± 8.9 (n=51)
MA	14 ± 17 (n=51)	<0.001	41 ± 18 (n=75)	0.003	30 ± 19 (n=51)
G	2370 ± 11113 (n=51)	ns	4198 ± 2539 (n=75)	ns	2804 ± 2524 (n=51)

Values are expressed as means ± SD.
P, significance (set at <0.05); ns, not significant; TEG, thrombelastograpy; Platelet, thrombocytes ($x10^9$/l); CCI, Corrected Count Increment; PT, prothrombin time (sec.); aPTT, activated partial thromboplastin time (sec.); AT, Antithrombin (%); Fibr., Fibrinogen (g/l); R, reaction time (min); K, clotting time (min); alpha, alpha angle (°); MA, maximum amplitude (mm); G, shear elastic modulus (dyne·cm^{-2}).

By TEG however, we observed a significant acceleration in the initiation phase of the plasmatic coagulation (decrease in R- & K-time) with also a higher rate of fibrin built up (higher alpha angle), and ultimately a clot with superior strength (higher MA), measured 1h after PLT transfusion. These pro-haemostatic effects could be measured until 24 hour after PLT transfusion, with only a trend in improvement for Reaction time. Further, we observed that it might take some time for stored PLTs to regain optimal function in circulation. In 21% of the transfusions Alpha, MA and G were significantly higher 24 hours after transfusion compared to 1 hour after transfusion;

this was not influenced by the storage time of the concentrates. Alpha 16 ± 15 versus 7 ± 6 degrees (p=0.01) versus 3 ± 2 before transfusion, MA 47 ± 17 versus 27 ± 22 mm (p=0.003) versus 17 ± 11 before transfusion and G 5251 ± 3105 versus 2519 ± 2376 dyne/cm^2 (p=0.005) versus 1090 ± 760 before transfusion; whereas platelet count decreased from 40 ± 12 x 10^9/L 1 hour after transfusion to 29 ± 14 x 10^9/L 24 hours after transfusion (p=0.0005). Noticeably, in 41 out of 92 study patients with severe thrombocytopenia, the initial TEG curve (before transfusion) showed a flat line, meaning that no initiation of coagulation could be measured at all.

Comparison of the haemostatic potential of native- and transfused-PLTs

The comparison of coagulation tests (classical and TEG) as measured in patients with different degrees of thrombocytopenia without any PLT transfusion (with only circulating patient's own, native PLTs) and during PLT transfusions (with most of the circulating PLTs being allogeneic), is shown in Table 2. With the exception of fibrinogen level, no statistically significant differences could be found in haemostatic potential between native PLTs and transfused blood bank stored PLTs (all p-values ≥0.1). Although clot initiation (R and K) and clot strength (MA) were similar for native- and transfused PLTs, there is a non-significant trend towards higher clot strength (MA) after PLT transfusion, compared to the effect on MA caused by native PLTs. The p-values for the difference in MA between both groups for the various platelet ranges are; PLT<25 x 10^9/L: p=0.4; PLT 25-50 x 10^9/L: p=0.12 and PLT 50-100 x 10^9/L: p=0.07.

Table 2. Classical coagulation tests and TEG parameters before and during PLT transfusions, for different ranges of PLTs.

PLT count	<25 x10^9/L		25-50 x10^9/L		50-100 x10^9/L	
Transfusion	before	during	before	during	before	during
N	10	15	16	51	8	21
PT	14 ± 2	14 ± 1	13 ± 1	14 ± 3	14 ± 1	12 ± 1
aPTT	30 ± 3	31 ± 3	30 ± 5	29 ± 5	27 ± 3	27 ± 3
AT	78 ± 29	94 ± 14	95 ± 20	96 ± 14	97 ± 11	100 ± 11
Fibr.	4 ± 2	5 ± 2	4 ± 1*	6 ± 2	6 ± 3*	4 ± 1
R	89 ± 48	90 ± 36	67 ± 40	63 ± 34	62 ± 67	46 ± 32
K	42 ± 8	55 ± 24	30 ± 13	30 ± 16	21 ± 13	19 ± 11
Alpha	4 ± 2	4 ± 4	8 ± 6	10 ± 8	16 ± 12	17 ± 9
MA	21 ± 14	26 ± 14	38 ± 17	45 ± 15	44 ± 19	54 ± 10

Values are expressed as means ± SD.
* *p,* significance (set at <0.05); PLT, platelet; TEG, thrombelastography; N, number included; PT, prothrombin time (sec.); aPTT, activated partial thromboplastin time (sec.); AT, Antithrombin (%); fibr., Fibrinogen (g/l); R, reaction time (min); K, clotting time (min); alpha, alpha angle (°); MA, maximum amplitude (mm).

Effect of storage time of PCs on haemostatic potential of PLTs

A comparison between the haemostatic effects of PCs with limited storage time to PCs with longer storage time on both classical coagulation tests and TEG parameters, measured before PLT transfusion, 1 hour after PLT transfusion and 24 hours after PLT transfusion, is shown in Table 3. Also in this table, native values for identical ranges of thrombocytopenia are presented between parenthesis. The transfusion of PLTs stored for 1-3 days (n=35, age 45±17 yr, 16 women, 19 men) demonstrated better pro-haemostatic effects then PLTs stored for 4-5 days (n=35, age 47±14 yr, 17 women, 18 men), measured 1 hour after transfusion. Both the time until a fixed level of clot firmness was reached (K-time; p=0.03) as well as the rate of clot growth (alpha angle; p=0.02) were superior for PCs with limited storage time (Figure 2). Although not significant, the same trend was observed 24 hour after transfusion, when the effects of fresh PLTs (n=23, age 45 ± 17 yr, 9 females, 14 males) were compared to the effects of older PLTs (n=21, age 47 ± 14 yr, 8 females, 13 males).

Importantly, although storage time might influence the haemostatic competence of PLT, both fresh and stored PLT demonstrated adequate haemostatic activity when compared to native (autologous) PLTs.

Table 3. Effect of fresh (vs. native) compared to stored (vs. native) platelets on classical coagulation tests and TEG parameters, measured 1 hour after PLT transfusion and 24 hours after PLT transfusion.

	Effect after 1 hour		Effect after 24 hour	
	Fresh (native)	Stored (native)	Fresh (native)	Stored (native)
PLT	41 ± 12 Range 16-72	34 ± 10 Range 14-50	29 ± 14 Range 2-56	23 ± 11 Range 3-42
CCI	19 ± 7	17 ± 6	12 ± 8	11 ± 6
PT	14 ± 1 (13 ± 1)	14 ± 1 (13 ± 1)	14 ± 1 (14 ± 1)	14 ± 1 (13 ± 1)
aPTT	28 ± 3 (31 ± 9)	29 ± 4 (32 ± 15)	28 ± 3 (33 ± 13)	29 ± 4 (31 ± 9)
AT	95 ± 13 (94 ± 20)	96 ± 14 (90 ± 21)	99 ± 13 (91 ± 33)	97 ± 15 (93 ± 20)
Fibr.	5 ± 2 (4 ± 1)	5 ± 2 (4 ± 1)	5 ± 2 (4 ± 1)	5 ± 1 (4 ± 1)
R	62 ± 42 (64 ± 36)	73 ± 40 (70 ± 35)	78 ± 49 (72 ± 35)	83 ± 55 (68 ± 36)
K	27 ± 16* (28 ± 13)	37 ± 22 (34 ± 13)	34 ± 21 (36 ± 14)	40 ± 24 (36 ± 15)
Alpha	13 ± 10* (10 ± 8)	8 ± 8 (7 ± 6)	9 ± 10 (6 ± 5)	8 ± 11(6 ± 5)
MA	46 ± 14 (39 ± 18)	40 ± 18 (33 ± 17)	35 ± 19 (28 ± 18)	31 ± 17 (28 ± 18)
G	4862 ± 2239 (3800 ± 2383)	4017 ± 2566 (2904 ± 2042)	3398 ± 2773 (2446 ± 1999)	2728 ± 2068 (2446 ± 1999)

Fresh =storage time of 1-3 days, Stored = storage time of 4-5 days. *Native* = autologous PLTs
Values are expressed as means ± SD.
* $p < 0.05$ for fresh vs stored PLT, by unpaired *t*-test; TEG, thrombelastography; PLT, platelets ($x10^9$/l); CCI, corrected count increment; PT, prothrombin time (sec); aPTT, activated partial thromboplastin time (sec) AT, antithrombin (%); fibr., fibrinogen (g/l); R, reaction time (min); K, clotting time (min); alpha, alpha angle (°); MA, maximum amplitude (mm); G, shear elastic modulus (dynes/cm^2).

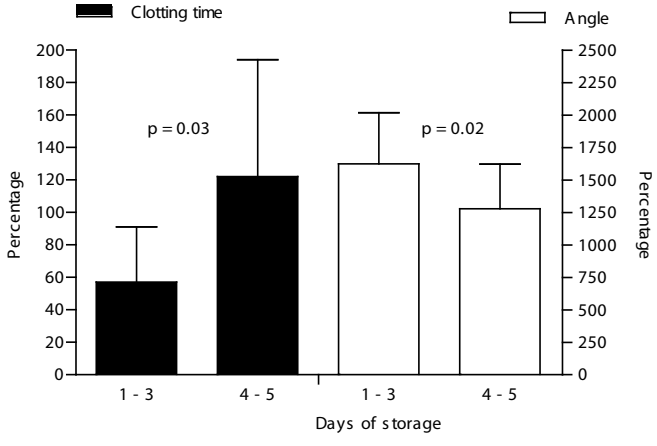

Figure 2.

Effect storage time of platelet concentrates on clotting time and alpha angle, 1 hour after transfusion, expressed as percentage (Mean ± SEM) from pretransfusion level.

DISCUSSION

In this study, we did not observe differences in haemostatic function between transfused (allogeneic) blood bank-stored PLTs and patient's own circulating native PLTs as measured by TEG. Moreover, we observed by TEG that transfusion of PCs with limited storage time (1-3 days) had superior haemostatic potential compared to transfusion of PCs with longer storage time (4-5 days).

The purpose of PLT transfusion is to supply a sufficient number of donor PLTs with haemostatic properties similar to those of the patient's own PLTs. Further, donor PLTs are supposed to remain their haemostatic capacity in the circulation for a substantial period of time, without clinical significant effects of prior storage time. The therapeutic effect of transfused PLTs cannot be predicted with certainty as storage, age and the recipient's condition alter the efficacy of PLT transfusion. In clinical practice the corrected count increment is used to quantify transfusion efficacy. Very few tests of PLT function performed on PCs in vitro correlate with PLT viability after transfusion and none has been adequately validated after transfusion (19). Classically, quality control analyses of PCs focus on PLT metabolism and energy state (20). In recent years, the testing of PLT function has shifted to the assessment of signaling processes and activation properties relevant to haemostasis. Although newer technologies like the use of flow chamber systems, thrombin generation assays and proteomics are promising, these techniques are time consuming to carry out and require specialized technical expertise (21-23). Thrombocytopenia primarily affects clot firmness (MA), but also the initiation phase of plasmatic coagulation (R- & K-time) as well as the speed of thrombin formation and fibrin built up (alpha angle), as monitored by TEG (13,15,24). In agreement with these studies, we observed a significant improvement of the initiation phase of plasmatic coagulation (decrease in R-&K-time) with also a higher rate of fibrin built up (higher alpha angle) and ultimately an improved in clot strength (higher MA) after PLT transfusion. These pro-haemostatic effects could be measured until 24 hour after PLT transfusion. Apart from the absolute PLT count and the derived corrected count increment, none of the other classical coagulation tests performed could detect any pro-haemostatic effect of the transfused PLTs. Remarkably, TEG could not detect initiation of coagulation in almost half of the

study patients with severe thrombocytopenia. The latter is in accordance to the findings of Johansson et al, who observed diminished or absence of clot formation in thrombocytopenic patients (PLT counts 10-20 x 10^9/L) who were 7 days post-stem cell transplantation (25). Although study populations are comparable, in the study of Johansson et al activated, recalcified citrated whole blood samples were used. However, despite the activation by tissue factor, clot formation was observed in only three out of 11 patients. This is in accordance to the concept that (functional) PLTs are essential to initiate and support thrombin generation. In clinical practice, where TEG is classically used as a rapid point of care test, activators of coagulation are often added to the TEG sample, which results into shortening of the initial part of the TEG line, making rapid interpretation of the coagulation process possible. However, as we demonstrated, this same activation may be at the cost of (subtle) information that can be obtained from the initial part of the TEG line (26). In this study on the haemostatic function of PLTs, we used TEG not because of its rapidity, but because we consider it an unique research tool as it offers an overview of the cumulative effects of all the individual components of haemostasis, providing information about the quality of the clot as well as the dynamics of its formation. By using this technique, we were able to demonstrate in a novel and unique way, comparable haemostatic function of circulating native PLTs and transfused, allogeneic PLTs. Further, by using TEG we were able to detect differences in haemostatic capacity between fresh and stored PCs in favor of fresh PCs. Noticeably this inferior haemostatic capacity for stored PCs was only significant for parameters related to the initiation phase of the coagulation process. Results from other studies on transfusion of fresh versus stored PCs showed that storage resulted in impairment of PLT viability in vivo, demonstrated by decreased PLT recovery and survival (27) and also in decreased PLT activation sensitivity (28). Moreover, in our study the observed haemostatic differences were no longer statistical significant 24 hours after the transfusion. The latter is in accordance to the findings of Owens and colleagues who found that stored PLTs may regain function in vivo, requiring at least 24 hours (29), as was also demonstrated in some of our study patients. The in vivo restoration of PLT function seems only beneficial in situations where PCs are prescribed for prophylaxis. In thrombocytopenic patients who show signs of active bleeding PCs with limited storage time might be more effective.

There were a number of limitations to this study. First, our study population was not healthy as patients in study group I had been treated for hematological malignancy with chemotherapy. As we did not include patients with active disease treated for with “induction” courses of chemotherapy, we tried to exclude any effect of the earlier disease on the coagulation process. Although we cannot exclude any (subtle) confounding effect of the (response on) treatment on study outcome, we assume that, due to our strict inclusion criteria, our study results and conclusions may be applicable to “normal” patients with thrombocytopenia who receive PLT transfusions. A second limitation is that whole blood samples without activators were used for TEG measurements as we wanted to use TEG as a research tool on (unmanipulated) whole blood as stated in our discussion section. However, the usage of activators resembles normal physiology as it is the TF pathway that provides the major stimulus for clot formation in vivo. Moreover, clinical evidence for clinical utility of TEG is mainly based on investigations using activators. As a consequence, the study observations may not apply to other methods of TEG measurement. Finally, as we measured only parameters of coagulation, but not the (clinical) effects of platelet transfusion on bleeding episodes (between study groups), we can only speculate on the clinical relevance of our findings.

In summary, we demonstrated that TEG was able to monitor the haemostatic effects of PLT transfusion with comparable haemostatic properties of transfused stored PLTs compared to native circulating PLTs. Further, our data suggest that limited storage time of PLTs is associated with better haemostatic capacity. However, before TEG can be applied as a clinically relevant qualitative test in PLT transfusion, further research is needed with focus on clinical outcomes like bleeding episodes.

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6

Effects of Red Blood Cells on Haemostasis

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Transfusion 2010;50:1536-1544

ABSTRACT

BACKGROUND: Currently there is no sensitive laboratory test to establish the influence of red blood cells (RBCs) on haemostasis. As thrombelastography® (TEG) measures haemostasis in whole blood, taking into account the interactions of all cellular elements, we used this instrument to investigate the role RBCs play in haemostasis.

STUDY DESIGN AND METHODS: In 29 patients with chemotherapy-induced anaemia we studied the effect of progressive anaemia on the coagulation profile. In 24 patients with chronic anaemia we studied the effect of transfusion of RBCs on coagulation. Finally, in 18 patients we evaluated whether storage time of RBCs has additional effects on haemostasis.

RESULTS: We observed a significant negative correlation between hemoglobin and TEG variables related to both cloth strength and elasticity ($p<0.05$). Moreover, anaemia was associated with a delay in the initiation of the coagulation cascade. Correction of anaemia by RBC transfusion resulted in significant shortening of this initiation phase with now the opposite effect on clot strength and elasticity. The negative effects on clot quality were significantly worse when fresh RBCs were transfused compared to longer stored RBCs. Furthermore, in contrast to the longer stored RBCs, fresh RBCs did not enhance initial fibrin formation.

CONCLUSION: In this study we found that anaemia was associated with a delay in the initiation of the coagulation cascade with a finally formed clot with superior strength and viscoelastic properties. Transfusion of RBCs was associated with impaired clot quality, with even worse effects on the initial fibrin build-up and clot quality by fresh RBCs.

INTRODUCTION

The role of platelets (PLTs) in haemostasis has been well established. This is in contrast to the possible role of red blood cells (RBCs) in haemostasis. Erythrocytes are involved in haemostasis as they flow in the center of the vessel pushing PLTs towards the site of action at the vessel wall and enhance shear forces, which activate PLTs. There is increasing evidence that RBCs not only have passive (rheological) effects on blood coagulation, but also actively stimulate both thrombin generation and PLT function in vitro (1-3) In addition, erythrocytosis is considered a risk factor for the development of thrombosis (4). The transfusion of RBC concentrates in anemic patients has been associated with a reduction in bleeding time (5,6). Further, a significant decrease in the activated partial thromboplastin time (aPTT) after RBC transfusions in patients with anaemia has been described (7).

Whether transfusion of RBCs with differences in storage time has similar effects on haemostasis is yet unclear. It is known that storage and blood banking procedures of RBCs have negative effects on viability and flow characteristics of the transfused erythrocytes (8). Berezina and associates reported that blood clotting progressively decreases after 2 weeks of storage, probably due to the exhaustion of some procoagulant plasma factor (9).

Currently there is no sensitive laboratory test to establish the potential impact of RBCs on haemostasis. To determine the relative contribution of the different components of haemostasis, conventional coagulation tests like bleeding time (BT), prothrombin time (PT), aPTT, fibrinogen concentration, and PLT count are measured. The limitation of these classical tests is that only information on the plasma coagulation factor levels is provided, but not on the potential physiological role of other cellular elements like RBCs.

Thrombelastography® (TEG) is a technology which can asses whole blood coagulation and offers a rapid overview of the cumulative effect of all the individual components of haemostasis, without having to analyze each of these components separately.

TEG provides information about the quality of the clot as well as the dynamics of its formation and its lysis. The different parts of the TEG tracing correspond to specific deficiencies in coagulation factors or inhibitors of coagulation, use of anticoagulants, PLT count as well as PLT function and fibrinogen level (10-12). TEG is classically used in situations where point-of-care testing of haemostasis is desired such as the perioperative setting. In trauma patients with penetrating injury as well as in patients after cardiac surgery, a reduction in TEG variable maximum amplitude (MA [clot strength]) has been more indicative of bleeding and transfusion requirements than "classical" tests of coagulation (13,14). Moreover, modifications of TEG allows the measurement of thrombin generation at different time points during the clot formation which is a clinical relevant approach to assess the global integrity of the thrombin complex (15,16).

As TEG measures haemostasis in whole blood, taking into account the interactions of all cellular elements, we decided to study the role of RBCs on haemostasis as measured by TEG in patients with different degrees of anaemia. In addition, we studied the effect of transfusion and of storage time of RBCs on haemostasis.

MATERIALS AND METHODS

Study patients

The institutional review board approved the study and informed consent was obtained from all patients and control persons. A total of 71 patients, divided over three groups were included in the study. In Group 1 we studied the effect of progressive anaemia on the coagulation profile. A total of 29 measurements were performed in 12 patients. All included patients were treated for hematological malignancies and admitted for their second or third course of chemotherapy. For all patients the underlying disease was in complete remission after the preceding chemotherapy, eliminating any effect of the disease on the coagulation process. Moreover, the bone marrow function had to be restored to normal levels with concomitant normal peripheral white blood cell (WBC) counts. The minimal PLT count had to be 150 x 10^9/l.

Patients included in Study Groups 2 and 3 suffered from chronic anaemia and needed RBC transfusion on a regular basis. The transfusion trigger was set at a Hb level of 7 to 8 g/L and patients were transfused with 2 to 3 units of RBC. We decided to study patients who were transfused with 3 units of RBC because we expected that the effect of RBC transfusion on haemostasis would be best measurable in this group. All transfusions were administered consecutively in a time interval of 6 hours. Group 2 consisted of 24 patients with chronic anaemia in whom we studied the effect of transfusion of three stored units of RBCs on coagulation. Finally, in Group 3 (18 patients), we evaluated whether storage time of RBCs has additional effects on haemostasis. All included patients in Group 3 were transfused with units of RBCs with a storage time of two days. In both Group 2 and Group 3, TEG and classical coagulation tests were performed before, 1 hour after, and 24 hours after the transfusion of 3 RBC units.

For all patients the following exclusion criteria were used: coagulation disorders, use of anticoagulants, use of acetylsalicylic acid within the past 10 days, use of non steroidal anti-inflammatory drugs within the last 24 hours, renal diseases or plasma concentration of creatinine more than 120 µmol/L and liver disease or increased plasma concentration of aspartate aminotransferase (ASAT; >50 U/L) or alanine aminotransferase (ALAT; >50 U/L). Patients with septicemia, diffuse intravascular coagulation, or thrombosis during the study period were also excluded.

Blood sampling and assays

Blood samples were obtained simultaneously for TEG analysis and standard laboratory and coagulation tests (i.e. complete blood count, WBC differentiation, creatinine, ASAT, ALAT, PT, aPTT, fibrinogen concentration and antithrombin [AT]). Venous blood samples were collected by vein puncture at the antecubital fossa using a 21-gauge butterfly needle. One single experienced examiner obtained all blood samples. To minimize the effects of venous endothelial damage by using a tourniquet, the first aspirate of 10 mL of blood was discarded. Blood was collected into a 20-mL polypropylene syringe to prevent contact activation of clotting by glass. Some of the collected blood, 3.5 mL, was filled into a tube (Vacutainer, Greiner Bio-One,

Kremsmünster, Austria), containing 0.5 mL of 3.2% Sodium Citrate for subsequent standard coagulation tests. All standard coagulation tests were performed on a coagulation analyzer (STA-R, Roche, Woerden, the Netherlands); PT and aPTT with reagents (Thromborel S and Actin FS, respectively, Dade Behring, Deerfield, IL), fibrinogen with excess thrombin (BioPool, Ventura, CA) according to the Clauss method and AT with thrombin as enzyme (STAchrome ATIII kit, Roche). Normal values for these variables in our laboratory are PT 11 to 16 sec, aPTT 26 to 36 sec, fibrinogen 1.7 to 3.5 g/L, and AT 75% to 125%.

TEG assays were carried out using a computerized TEG coagulation analyzer (Model 5000; Haemoscope Corp., Niles, IL). All analyses were performed with TEG disposable cups and pins as devised by the manufacturer. Polypropylene and polyethylene pipettes were used to handle reagents and blood. TEG analyses were performed in native whole blood, without activator. Adding activators to the TEG sample results into shortening of the initial part of the TEG line, making rapid interpretation of the coagulation process possible. However, this same activation may be at the cost of (subtle) information from this initial part of the TEG line (17). To maximize the information that can be obtained by TEG, we used nonactivated whole blood. For TEG analysis in native whole blood, 360 µL of whole blood was pipetted into the prewarmed TEG cup and measurements were performed within 6 minutes of sampling (18). The following TEG variables were recorded (in parenthesis normal values expressed as mean ± SD obtained in our laboratory in 120 normal persons): the reaction time (R-time, 22 ± 5 min), representing the rate of initial fibrin formation; the clotting time (K-time, 9 ± 3 min), representing the time until a fixed level of clot firmness is reached; the angle (α, 26 ± 8 degrees), which is closely related to K-time and represents the rate of clot growth; the maximum amplitude (MA, 46 ± 7 mm), is a measurement of maximum strength or stiffness of the developed clot; the shear elastic modulus strength (SEMS or G, 4506 ± 1392 dyne cm^{-2}) is a parametric measure of clot firmness expressed in metric units calculated from MA as follows:

$$G = (5000 \times MA) / (100 - MA).$$

R-time, K-time and α are prolonged by anticoagulants and factor deficiencies; MA is especially influenced by PLT and PLT function as well as fibrinogen level.

In addition to the former "classical" TEG variables we made velocity calculations describing thrombus generation during blood clotting that are derived from the signature graph produced by TEG. The following Thrombus generation (TG) parameters were recorded: the Maximum Thrombus Generation (MTG, 4.1 ± 1.3 dynes/cm^2/sec); this variable presents the first derivate of the velocity of the increase in clot strength, beginning as G begins to increase and ending after clot strength stabilizes. The information from this variable is equivalent to the information from the α angle, however MTG provides a more parametric evaluation than the determination of α. The second variable is Time to Maximum rate of thrombus Generation (TMG, 1747 ± 693 sec), which is the time it takes to reach MTG. Finally we determined Total Thrombus Generation (TTG, 769 ± 114 dynes/cm^2), which is the total positive area under the velocity curve, representing the total change in elastic resistance until clot strength stabilization occurs. MTG and TTG are expressed using metric units of elastic resistance that accurately describe changes in clot strength.

Statistical analysis

For statistical analysis SPSS computer software (SPSS 16.0 software for Windows, SPSS, Inc., Chicago, IL) was used. Variables are expressed as mean ± SD. The correlations of changes in Hemoglobin (Hb) level and individual TEG variables were calculated using Pearson's correlations. Mean values of TEG and classical coagulation variables were tested by an unpaired t test comparing patients with Hb levels of less than 10 g/dL with patients with Hb levels of more than 10 g/dL. To study the effects on coagulation variables 1 and 24 hours after blood transfusion, a paired samples t test was used. Significance levels were set at 0.05 (two-tailed).

RESULTS

Effect of anaemia on coagulation

In group 1 we studied the relationship between Hb level and coagulation variables. Patient demographics are summarized in Table 1. In this study group a total of 29 measurements were performed in 12 patients, of which four were treated for multiple myeloma, seven for acute leukemia and one for Hodgkin's lymphoma. Due

to more intensive induction chemotherapy in patients with acute leukemia these patients entered this part of the study with a lower Hb level. To compare the effects of higher Hb levels with lower Hb levels we chose the cutoff Hb of 10 g/dL to obtain groups of equal size. Due to the lower age of acute leukemia patients compared to patients with multiple myeloma and to the lower Hb level with which acute leukemia patients entered this part of the study the age of patients in the group with Hb levels of less than 10 g/dL in Table 1 is considerable lower compared to the age in the groep with Hb levels of more than 10 g/dL. We found that Hb level was significantly negatively correlated with the classical coagulation test PT ($r = -0.7$; $p<0.001$) and TEG variables related to both cloth strength (MA, MTG) and clot elasticity (SEMS; $r = -0.4$, $p<0.05$). Moreover, we observed that anaemia was associated with a higher rate of fibrin built up (increase in α angle). A decreasing Hb level was associated with prolongation of R-time, suggesting a delay in the initiation of the coagulation cascade in anemic patients. As expected there was a significant positive correlation between the classical coagulation test PT and TEG variable R-time. The most illustrative scatter diagrams and regression equations of classical coagulation test PT and TEG variables R, MA and MTG versus Hb level are presented in Figures 1a-d.

Table 1. Basic demographic data of included patients

Study group:	**Group 1 progressive anaemia**		**Group 2 RBC transfusion, > 2 days' storage**		**Group 3 RBC transfusion, 2 days' storage**	
	Hb <10g/dL	**Hb >10g/dL**	**1 hr**	**24 hr**	**1 hr**	**24 hr**
Number	15*	14†	24	24	18	18
Age (yr)	24 ± 9	46 ± 17‡	51 ± 18	53 ± 17	49 ± 14	50 ± 15
male/female	4 / 11	4 / 10	12 / 12	12 / 12	12 / 6	13 / 5

* Fifteen measurements in 5 patients; 4 with acute leukemia and 1 with Hodgkin's lymphoma.
† 14 measurements in 7 patients: 4 with multiple myeloma and 3 with acute leukemia.
‡ $p<0.05$.

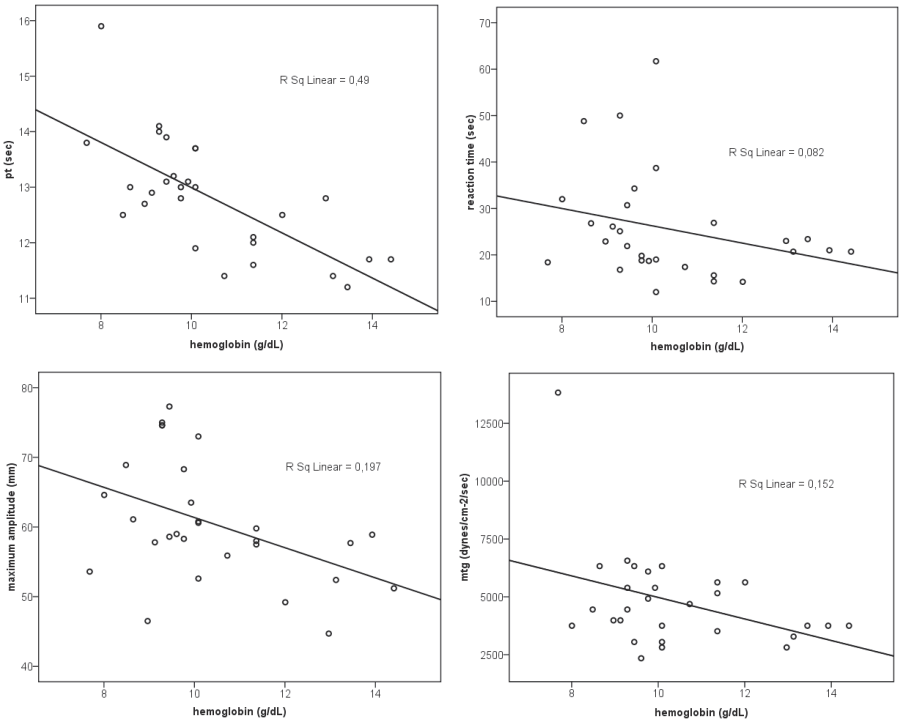

Figure 1a-d.

Correlation and linear regression of PT, R-time, Maximum Amplitude and MTG with Hb level.

We also divided Group 1 into two groups of equal size; one group with a “lower” Hb and another group with a “higher” Hb. We chose a cutoff level for Hb of 10 g/dL because this was both the mean and the median Hb level of the entire cohort. Significant differences in PT (p=0.01), clot strength (MA; p=0.02), and viscoelastic properties of the formed cloth (SEMS; p=0.02) were found when patients with Hb levels less than10 g/dL were compared to patients with Hb levels of more than10 g/dL. Box plots for these three variables are shown in Figures 2a-c.

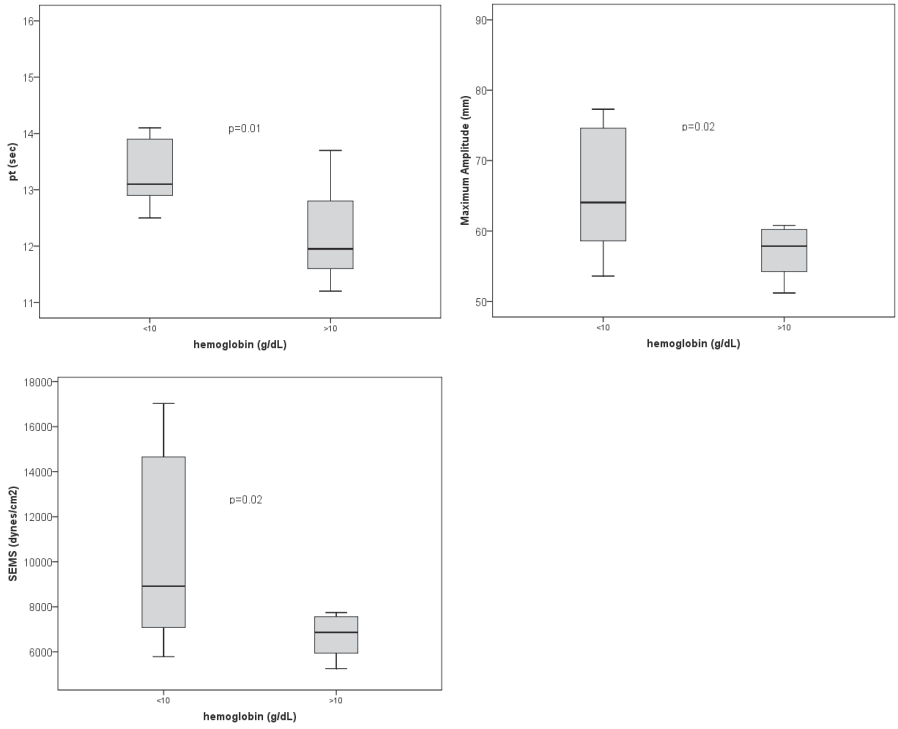

Figure 2a-c.

Box plots of the variable PT, Maximum Amplitude and SEMS comparing patients with Hb levels of less than 10 g/dL with patients with Hb levels of greater than 10 g/dL.

Effect of transfusion of stored RBC on coagulation

In study Groups 2 and 3 we studied the effect of transfusion of stored and "fresh" RBCs on coagulation. The basic demographics of both study groups are demonstrated in Table 1. The percentage change in coagulation parameters 1 and 24 hours after transfusion of 3 units of RBCs with a mean storage time of 18 ± 7 days is shown in Table 2. It demonstrates that 1 hour after transfusion, the initiation of plasmatic coagulation is accelerated, illustrated by a significantly shortened R-time. This results, however, in a clot with inferior strength and quality as is demonstrated by a decrease in the TEG variables MA, SEMS, MTG and TTG. This negative effect on clot quality even persisted 24 hours after the transfusion. From the classical coagulation tests, a significant increase in aPTT was observed during the first hour after transfusion.

Effect of transfusion of fresh RBCs on coagulation

Table 3 shows the effects on coagulation parameters 1 and 24 hours after the transfusion of 3 units of RBCs with a limited storage time of 2 days. A significant decrease in clot strength and clot quality (TEG variables MA, SEMS, and TTG) was observed 1 and 24 hours after the transfusion. From the classical coagulation tests, a significant increase in aPTT was observed 1 hour after transfusion. Compared to the results obtained with the longer-stored RBC's, MA and SEMS are significantly more reduced (MA 1 and 24 hr, respectively, p=0.003 and p=0.002; SEMS 1 and 24 hr, respectively, p=0.02 and p=0.004), suggesting an even greater decrease in clot strength and quality when fresh RBCs are used (Tables 2 and 3, Figure 3).

Table 2. Exact baseline values and changes (%) in classical and TEG coagulation variables after transfusion of RBCs with storage time of 18 ± 7 days

	Exact baseline values	% change after 1hour	*p*	% change after 24 hour	*p*
Hb	8.2 ± 0.7	135 ± 8	<0.0001	140 ± 7	<0.0001
Platelet	144 ± 98	90 ± 12	0.004	89 ± 14	0.024
PT	13.6 ± 1.5	99 ± 3	0.241	98 ± 3	0.013
aPTT	28.1 ± 3.1	103 ± 5	0.001	111 ± 25	0.116
Fibr	4.0 ± 1.0	103 ± 11	0.244	106 ± 13	0.114
AT	99.4 ± 17.2	100 ± 8	0.871	102 ± 6	0.218
R	36.7 ± 21.3	83 ± 36	0.004	92 ± 50	0.465
K	13.7 ± 12.0	113 ± 71	0.315	152 ± 155	0.324
α	28.5 ± 17.4	108 ± 38	0.713	108 ± 50	0.452
MA	61.5 ± 12.8	92 ± 7	<0.0001	91 ± 18	0.025
SEMS	9358 ± 4424	80 ± 14	<0.0001	83 ± 35	0.014
MTG	5.7 ± 3.9	97 ± 40	0.004	94 ± 51	0.206
TMG	4372 ± 1647	88 ± 42	0.143	104 ± 67	0.762
TTG	1013 ± 224	92 ± 6	<0.0001	93 ± 14	0.0020

Table 3. Exact baseline values and changes (%) in classical and TEG coagulation variables after transfusion of RBCs with storage time of 2 days.

	Exact baseline values	% change after 1hr	*p*	% change after 24hr	*p*
Hb	8.3 ± 0.7	135 ± 10	<0.001	137 ± 13	<0.001
PLT	181 ± 136	90 ± 11	0.004	82 ± 22	0.001
PT	14.1 ± 1.1	99 ± 2	0.117	98 ± 5	0.165
aPTT	31.2 ± 5.4	105 ± 8	0.019	104 ± 8	0.102
Fibr	5.3 ± 2.0	102 ± 7	0.333	99 ± 7	0.266
AT	107.4 ± 21.1	103 ± 8	0.135	107 ± 22	0.429
R	36.1 ± 15.4	106 ± 52	0.256	107 ± 47	0.301
K	13.1 ± 5.7	113 ± 40	0.241	134 ± 60	0.161
α	20.8 ± 11.9	94 ± 38	0.543	82 ± 43	0.039
MA	61.9 ± 18.8	82 ± 13	<0.001	75 ± 23	<0.001
SEMS	10768 ± 6835	67 ± 19	<0.001	55 ± 18	<0.001
MTG	4.9 ± 2	89 ± 25	0.116	72 ± 28	0.034
TMG	3117 ± 1418	107 ± 73	0.669	100 ± 44	0.781
TTG	1070 ± 224	87 ± 11	<0.001	82 ± 11	0.347

Hb; hemoglobin, PT, partial thromboplastin time; aPTT, activated partial thromboplastin time; AT, anti-thrombin, Fibr; fibrinogen, R-time, reaction time; K-time, clotting time; α, alpha angle; MA, maximum amplitude; SEMS, shear elastic modulus strength; CI, coagulation index; MTG, maximum thrombus generation; TMG, time to maximum thrombus generation; TTG, total thrombus generation.

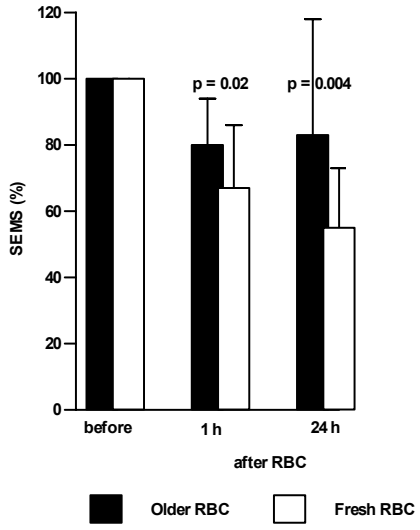

Figure 3.

SEMS 1 and 24 hours after transfusion of 3 RBC units expressed as percentage of SEMS before transfusion. (■) Older RBCs; (□) fresh RBCs.

DISCUSSION

In this study we observed that RBCs indeed play a role in haemostasis. In case of decreasing Hb level, we observed a significant improvement in both clot strength and quality, as was demonstrated by the finding of an increase in TEG variables MA, SEMS, and TMG. However, this phenomenon was accompanied by a tendency towards a delay in the initiation phase of plasmatic coagulation, illustrated by an increase in both PT and R-time.

Further, we found that correction of anaemia by RBC transfusion resulted in significant shortening of the initiation phase of plasmatic coagulation (decrease in R-time) with now the opposite effect on clot strength and clot elasticity (decrease in MA, SEMS, MTG and TTG). These negative effects on clot strength and elasticity persisted until 24 hours after RBC transfusion. Transfusion of RBCs with storage time of 2 days showed a significantly stronger negative effect on clot strength and

quality. Remarkably, with these fresh RBCs no significant (procoagulant) effect on the initiation phase of plasmatic coagulation (R-time) was observed.

Translated into terms of coagulation we found that in case of anaemia the initial rate of fibrin formation seems to be delayed, until a certain amount of thrombus was generated. From that point on, the coagulation profile was superior in terms of a more rapid fibrin build-up with finally a stronger clot with better viscoelastic properties.

Correction of anaemia by RBC transfusion, on the other hand, showed exactly the opposite effects on the coagulation profile. After transfusion the initiation phase of the plasmatic coagulation was shortened with deterioration of the ultimate clot strength and quality. Although haemostatic effects could be seen with both stored and fresh RBCs, the detrimental effects on haemostasis were more obvious for fresh RBCs compared to stored RBCs.

In group 1, patients with Hb levels below 10 g/dL were significantly younger than patients with Hb levels above 10 g/dL (Table 1). Apart from age, no significant differences in gender were found, neither was there is a significant difference in PLT count between the two groups. It is know that aging affects the coagulation profile as measured by TEG (19). If age might have affected our study results, the true (prothrombotic) effect of anaemia on TEG variables may have been underestimated as aging usually is accompanied by a trend towards hypercoagulability.

It is known that bleeding time is prolonged in anemic patients, independent of their PLT count, and is shortened by elevating the hematocrit (Hct). The initial phase of haemostasis consists of PLT adhesion to subendothelial collagen, their activation and aggregation and finally the formation of a PLT plug. Lateral migration of RBCs in flowing blood contributes to this process, as it induces near-wall excess of PLTs (1). Also procoagulant phospholipids exposed on the outer surface of erythrocytes can serve as an trigger for the initiation of the coagulation cascade (20). The relation between Hct and coagulation is examined in a few other studies. A state of relative

hypercoagulability (decrease in R-time) was found immediately after a rapid 10% loss in circulating blood volume, probably related to rapid immediate hemodilution (21). Our findings are only partially in accordance with the findings of Iselin and co-workers (22). In their in vitro study, a decrease of the Hct from 40% to 10% resulted in a shortening of R- and K-time, with an increase in α angle, MA, and G, representing acceleration of blood coagulation with low Hct values. They concluded that the isolated reduction in Hct did not compromise in vitro blood coagulation. The finding of a faster initiation of the plasmatic coagulation process might be related to their study procedure in which RBCs were diluted with various amounts of 0.9% saline and resuspended into PLT- rich plasma in order to obtain samples with different Hct values. Another study observed a procoagulant state with mild saline hemodilution in vivo (23). They found a shortened R-time and K-time and widened α angle at levels of hemodilution of 20% to 30%. A disproportionate reduction in concentrations of natural anticoagulants such as AT with hemodilution has previously been suggested as a possible explanation (5,21). More recently, an inverse correlation was found in vitro between Hct and closure time as measured with the Platelet Function Analyzer (PFA-100®, Dade Behring) (24). This observation is in agreement with our finding of an inverse correlation between Hct and R-time. In contrast to the TEG technique, which gives insight into the total coagulation process, the PFA-100 only gives information about primary haemostasis. Therefore, no effects on clot strength and clot firmness could be measured. Although TEG variable MA is considered as an equivalent of bleeding time, as both give information on PLT count and function, our finding of improved clot strength (higher MA) is not in accordance to earlier observations of increased bleeding time in anemic patients.

In our study we observed that transfusion of RBC led to an improvement in the initial rate of fibrin formation, as was demonstrated by a significant decrease in R-time. However, the latter was accompanied by a significant negative effect on clot strength and clot elasticity. All these haemostatic effects could be measured immediately after the transfusion of RBCs and lasted for at least 24 hours. Although a positive relation was found between PT and R-time, no significant effect on PT was observed in the first 24 hours after transfusion of RBCs.

We also examined the influence of storage time of RBCs on the coagulation profile after transfusion. It is known that RBCs undergo lesions upon storage, which affect their function and possibly clinical outcome (25). Storage of RBCs also leads to the loss of RBC 2,3-diphosphoglycerate with impaired oxygen release during the first hours after transfusion. In a study performed in critically ill patients, RBC transfusions were independently associated with an increased mortality (25,26). In the CRIT study however, mortality was not increased in patients receiving old RBCs (>14 days stored) versus fresh RBCs. Others however reported that the transfusion of RBCs older than 7 days may contribute to hemorheologic disorders in critically ill patients (9). They observed a decrease in blood clotting time, measured by Sonoclot coagulation analyzer (Sienco, Inc., Färgelanda, Sweden), starting after 2 weeks of storage. It is, however, noticeable that, in contrast to our study, the latter study was performed in vitro. In our in vivo study, transfusion of both stored and fresh RBC led to a decrease in ultimate clot strength and clot quality. Moreover, in contrast to fresh RBCs, the transfusion of stored RBCs had beneficial effects on the initiation of plasmatic coagulation as R-time was significantly shortened. Further, both clot strength and clot quality seemed to be more compromised after transfusion of fresh RBCs compared to transfusion of stored RBCs. The inferior haemostatic function of fresh RBC's might be related to the manufacturing process of RBCs. Centrifuge speed may also have an impact on blood cell damage (27,28). It is conceivable that high centrifugal forces, used to separate whole blood into its components, and leukofiltration induce damage, which only slowly recovers due to the small amount of plasma left and the use of the additive solution saline-adenine-glucose-mannitol (SAGM). The difference in haemostatic effect of old versus fresh RBC could also be related to the effects of SAGM. Gulliksson and colleagues (29) observed during storage of RBC in SAGM an increased thrombin acitivity measured by fibrinopeptide A determination especially at a storage time longer than 3 weeks. However, provided the observed effect of RBC transfusion on haemostasis is of clinical relevance, it is important to state that the primary goal of RBC transfusion is to improve oxygen delivery. In this context, (possible) effects on haemostasis can often be easily resolved by adding PLTs and/or fibrinogen.

There were a number of limitations to this study. First of all, our study identifies associations between changes in Hb level variables of coagulation as measured by TEG and classical coagulation tests. Although the associations observed showed significance, no causality was demonstrated. Importantly, as we measured only variables of coagulation, but not the (clinical) effects of anaemia and RBC transfusion on bleeding episodes, we can only speculate on the clinical relevance of our findings. Moreover, our study population was not healthy as patients in study Group 1 had been treated for haematological malignancy with chemotherapy, As we did not include patients with active disease treated for with "induction" courses of chemotherapy, we tried to exclude any effect of the earlier disease on the coagulation process. Importantly, all included patients had normal values for PLT count and fibrinogen and antithrombin levels, making low-grade disseminated intravascular coagulation or consumptive coagulopathy very unlikely. Although we cannot exclude any (subtle) confounding effect of the (response on) treatment on study outcome, we assume that, due to our strict inclusion criteria, our study results and conclusions may be applicable to "normal" patients with different degrees of anaemia. Further, hemodilution as well as the effects of anticoagulants in the blood products could both have played a role in the first hours after transfusion; although only small amounts of plasma (10–20 mL/U) and anticoagulants are present in leukoreduced RBCs stored in SAGM. However, we observed identical haemostatic effects 1 and 24 hours after blood transfusion, making the effects of hemodilution and the effects of anticoagulants less likely. Another limitation is that whole blood samples without activators were used for TEG measurements. As a consequence, the study observations may not apply to other methods of TEG measurement. Also the additional measurement of TEG fibrinolysis variable(s) could have been of great interest, especially with regard to the qualitative influence of thrombin generation on clot resistance to fibrinolysis (30).

In summary, in this study we observed that lower Hb levels were associated with improvement in TEG variables corresponding to clot strength and elasticity, but also with a delay in the initiation of the coagulation cascade. Further, we observed that correction of anaemia by transfusion of RBCs seemed to have the opposite effects

on the coagulation profile, with shortening of the initiation phase of coagulation, but at the cost of inferior cloth strength. Stored and fresh RBCs both showed effects on clot quality, with even worse effects on the initial fibrin built up and clot quality by fresh RBC. Future studies on outcome (i.e., bleeding episodes) are needed to further elucidate the (clinical) role RBCs play in haemostasis.

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7

Thrombelastography can detect hypercoagulability and monitor the effects of Hydroxyurea in patients with Sickle Cell Disease

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submitted

ABSTRACT

BACKGROUND AND OBJECTIVES: The pro-coagulant profile observed in patients with Sickle Cell Disease (SCD) is complex and multifactorial. Thrombelastography (TEG) has the advantage of integrating all the aspects of coagulation into one single test. We used TEG to analyze hypercoagulability in patients with SCD, both in steady state and in painful crisis. Further, we studied the effects of Hydroxyurea (HU) and the role of red- and white- blood cells on haemostasis in SCD.

PATIENTS AND METHODS: In 18 patients with SCD (11 on HU), coagulation parameters could be measured in steady state and during 34 painful crises. Coagulation parameters in SCD patients were compared to controls and steady state was compared to crisis, in relation to HU use.

RESULTS: We observed statistically significant pro-coagulant TEG parameters in the SCD population not using HU compared to the control group both in steady state and in crisis. Apart from the ultimate clot strength, HU significantly reduced hypercoagulability compared to both controls and HU non-users. Further, both red and white blood cells were respectively negatively and positively correlated to hypercoagulabity.

CONCLUSION: In patients with SCD, TEG might be a useful tool to both identify- and monitor- for hypercoagulability as well as to detect the effects of treatment with HU.

INTRODUCTION

Patients with sickle cell disease (SCD) have chronically activated coagulation (1). The pro-coagulant state in SCD is considered multifactorial, involving haemostatic changes, activation of the coagulation cascade and endothelial activation (2,3). Although the activating effects of SCD on the coagulation system have been studied intensively, a unifying mechanism explaining this phenomenon has been elusive (4). Reported mechanisms include increased generation of both thrombin and fibrin, increased platelet and tissue factor activity, alterations in markers of coagulation activation and natural anticoagulant proteins and thrombophilic DNA mutations (5-9). Classically, SCD-related complications are thought to be caused by micro vascular occlusions and ischemic tissue necrosis following the adhesion of red blood cells (RBCs) and other cellular elements to vascular endothelium and the polymerization of sickle hemoglobin. More recently, interest has shifted toward the effects of intravascular hemolysis, leading to increased plasma concentrations of microparticles and the release of both cell-free hemoglobin and RBC arginase resulting into impaired nitric oxide (NO) bioavailability resulting into endothelial dysfunction and vasculopathy (10,11).

Thrombelastography (TEG) has gained popularity as a global point of care test of haemostasis and has the advantage over conventional tests of haemostasis that it is performed on whole blood, taking into account the role of interacting blood elements such as phospholipid bearing cells and platelets (12). The technique offers a rapid overview of the cumulative effect of all the individual components of haemostasis, without having to analyze each of these components separately, defining it as a more physiological instrument to study haemostasis (13,14). Moreover, TEG provides information about the quality of the clot as well as the dynamics of its formation and its lysis. There is also increasing clinical interest in assessment of the prothrombotic tendency by TEG, for example in thrombophilia screening, but also in prediction of arterial or venous thrombosis in the general population (15). TEG is considered more sensitive than routine assays in detecting hypercoagulability (16-19).

As a consequence, we hypothesized that TEG might be a useful tool to further elucidate the complex derangements in haemostasis in SCD. In the present study we used TEG to analyze hypercoagulability in patients with SCD, both in steady state and in painful crisis. Further, we studied the role of red and white blood cells as well as the effects of Hydroxyurea (HU) on haemostasis as measured by TEG in SCD.

MATERIALS AND METHODS

Study patients

The institutional review board approved the study and informed consent was obtained from all patients. Study patients were recruited from the SCD population visiting the outpatient department. 11 of the 18 included patients were on HU therapy. Patients were classified as having a painful crisis if symptoms consistent with a vaso-occlusive episode were present leading to hospital admittance.

In order to obtain our own normal (reference) values, we performed TEG and classical coagulation tests in 120 healthy adults; 60 women and 60 men, ten per decade, age distributed equally between 19 and 87 years, mean age 50 ± 17 years (20). From this cohort we selected an age matched control group.

For study patients and controls, the following exclusion criteria were used: coagulation disorders, use of anticoagulants, use of acetylsalicylic acid within the preceding 10 days or use of non steroidal anti-inflammatory drugs within the last 24 hours.

Study protocol

In patients with SCD TEG measurements and blood laboratory tests were performed in steady state at a routine visit to the outpatient department. During admittance for painful crisis, several serial TEG measurements and blood laboratory tests were performed until the crisis resolved. In the age matched control group the same TEG measurements and standard coagulation tests were performed. TEG and coagulation tests comparisons were made between steady state and crisis in the study group with SCD, and between the study group and the control group. Also, in the study

group with SCD, coagulation effects of HU were evaluated by comparing patients using HU with HU non-users.

Blood sampling and assays

In both the study and control group blood samples were obtained simultaneously for TEG analysis and standard laboratory- and coagulation-tests (i.e. the complete blood count, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen and antithrombin (AT). Moreover, in the study group reticulocyt count, lactate dehydrogenase (LDH), total billirubin, fetal hemoglobin level (HbF), factor VIII concentration (FVIII) and haptoglobin level were tested as parameters of SCD activity. Venous blood samples were collected by vein puncture at the antecubital fossa using a 21-gauge butterfly needle. Two examiners obtained all blood samples, both experienced in performing phlebotomy. Blood was collected into a 20-mL polypropylene syringe to prevent contact activation by glass. To minimize the effects of using a tourniquet, the first aspirate of 10 blood was discarded. All standard coagulation tests were performed on the STA-R coagulation analyzer (Roche, Diagnostica Stago, Asnières, France): PT with Thromborel S reagents and aPTT with Actin FS reagents (Dade Behring, Marburg, Germany), fibrinogen with excess thrombin (BioPool, Umea, Sweden) according to the Clauss method, and AT with thrombin as enzyme (STAchrome ATIII, Roche kit). Normal values for these variables in our laboratory are PT 11 to 16 sec, aPTT 26 to 36 sec, fibrinogen 1.7 to 3.5 g/L, AT 75% to 125%.

Thrombelastographic assays were carried out using a computerized TEG coagulation analyzer (Model 5000; Haemoscope Corp., Niles, IL). All analyses were performed with TEG disposable cups and pins as devised by the manufacturer. Polypropylene and polyethylene pipettes were used to handle reagents and blood. For TEG analysis 360 mL whole blood was pipetted into the prewarmed TEG cup and measurements were performed within 6 minutes from sampling (16). No activators of coagulation were added to the samples. The following TEG parameters were recorded: the reaction time (R), representing the rate of initial fibrin formation; the clotting time (K), representing the time until a fixed level of clot firmness is reached; the angle

(α), representing clot growth and correlated to thrombin generation which results into interaction between platelets and fibrin and, finally, the maximum amplitude (MA), which is a measurement of maximum strength or stiffness of the developed clot. the R time, K time and α are prolonged by anticoagulants and coagulation factor deficiencies, MA is especially influenced by platelet count and platelet function as well as fibrinogen level.

Statistical analysis

For statistical analysis SPSS 17.0 software for Windows was used. TEG and classical coagulation variables are presented as mean and SD. Group comparisons were made by an unpaired t-test. Significance levels were set at 0.05 (two-tailed). Linear regression was used to quantify the associations of TEG variables with relevant blood test. In these analyses, Pearson correlation coefficients (r^2) were calculated, as well as their level of significance (with a null hypothesis $r = 0$).

RESULTS

We studied 18 patients with SCD; the demographics of the study and control population are presented in Table 1. Of the 18 included patients, 11 were on HU treatment. Coagulation tests could be performed in all included patients during steady state of the disease. During admittance because of painful episode (=crisis), a total of 34 measurements of coagulation could be performed; 20 in patients on HU and 14 in patients without HU treatment.

Coagulation tests in SCD in steady state

Table 2 demonstrates TEG variables and classical coagulation test in patients with SCD in steady state compared to the same tests performed in healthy controls. The SCD cohort was divided into HU users and HU non users. We observed significant pro-coagulant parameters in the SCD population not using HU compared to the control group. Both the initial fibrin formation (R-time, p=0.02) and the time until a fixed level of clot firmness was reached (K-time, p=0.008) as well as the rapidity of clot growth (alpha angle, p<0.001) and maximum strength or stiffness of the developed

clot (Maximum Amplitude, p<0.001) were significant pro-coagulant in the HU naïve cohort.

Table 1. Demographics of study- and control- population

	Sickle Cell Disease HU -	HU +	Control Group
Number included	7	11	40
Mean age ± SD (yr)	33 ± 18	25 ± 7	29 ± 6
Gender (female: male)	5 : 3	3 : 8	21 : 19
Hb F (%)	3.8 ± 2.6	14.6 ± 7.3	-
Hb (mmol/l)	5.9 ± 1.3	6.3 ± 0.8	8.6 ± 0.8
Leuco (x10^9/l)	11.4 ± 2.9	6.9 ± 2.4	7.3 ± 1.4
Trombo (x10^9/l)	332 ± 74	346 ± 142	246 ± 48

HU -; Hydroxyurea non-user, HU +; Hydroxyurea user, Hb F; Fetal Hemoglobin, Hb; hemoglobin, Leuco; leucocytes, Trombo; trombocytes

Table 2. Comparison of TEG- and coagulation- parameters between control group and patients with SCD, in steady state*

TEG- & Coagulation-parameters	Control Group	p	SCD: HU – HU +
R-time (min)	22.2 ± 4.8	0.02 ns	17.4 ± 5.3 23.4 ± 8.0
K-time (min)	9.7 ± 2.7	0.008 ns	6.5 ± 3.4 8.5± 2.4
Alpha (degree)	23.0 ± 5.9	<0.001 ns	36.3 ± 18.2 25.5 ± 4.8
MA (mm)	42.6 ± 5.4	<0.001 0.01	62.7 ± 10.7# 47.4 ± 7.6
Trombo (x10^9/l)	245 ± 48	<0.001 <0.001	332 ± 74 346 ± 14
PT (sec)	12.6 ± 0.7	<0.001 <0.001	13.8 ± 0.8 13.8 ± 0.8
APTT (sec)	32.0 ± 3.2	0.006 ns	28.2 ± 3.5 30.9 ± 3.2
Fibrinogen (g/l)	2.7 ± 0.5	<0.001 ns	3.5 ± 0.7# 2.8 ± 0.4
AT (%)	105 ± 10	ns ns	102 ± 13 108 ± 10

* Displayed values are mean ± standard deviation. # p<0.05 HU- vs. HU+
SCD: Sickle cell disease HU -; Hydroxyurea non-user, HU +; Hydroxyurea user.
R-time; reaction time, K-time; clotting time, Alpha; alpha angle, MA; maximum amplitude. Trombo; trombocytes, PT; prothrombin time, APTT; activated partial tromboplastin time, AT; antithrombin, ns; not significant.

Interestingly, in HU users we observed that, apart from the ultimate clot strength, TEG parameters differed not statistically significant from the TEG parameters observed in the control group. Moreover, when HU users were compared to non HU users we found that the latter not only had significant higher ultimate clot strength (Maximum Amplitude, p=0.003) but also higher fibrinogen levels (p=0.02) with trends for hypercoagulabitiy for all other TEG parameters.

Coagulation tests in SCD in crisis

Table 3 demonstrates TEG parameters and classical coagulation tests performed in 34 painful episodes compared to the same tests performed in controls. A statistically significant procoagulant profile for all TEG parameters was observed in the HU naïve group compared to controls. Although in the group using HU the pro-coagulant patterns was somewhat weaker, patients still demonstrated significant higher initial fibrin formation (R-time; p=0.02) as well as a higher ultimate clot strength (Maximum Amplitude; p<0.001) compared to controls.

When HU users were compared to non HU users we found that HU naïve patients not only had significant higher initial fibrin formation (R-time; p=0.006) but also demonstrated a more rapid clot growth (Alpha angle; p=0.04) with trends for hypercoagulabitiy for the other TEG parameters.

When TEG parameters during crisis were compared to those in steady state, although not significant, a further increase towards hypercoagulability was observed for all TEG parameters in both HU users and HU naïve patients. From the classical coagulation test, platelet count significantly decreased during crisis, possibly caused by consumption (data not separately shown). A graphical presentation of our findings is demonstrated in Figure 1. Bar charts in this figure represent TEG parameters (+95% CI) in HU users and HU non-users in both steady state and crisis.

Table 3. Comparison of TEG- and Coagulation- parameters between control group and patients with SCD, in crisis*

TEG- & Coagulation-parameters	Control Group	p	SCD: HU – HU +
R-time (min)	22.2 ± 4.8	<0.001 0.02	14.4 ± 6.7# 18.9 ± 5.9
K-time (min)	9.7 ± 2.7	<0.001 ns	4.9 ± 3.0 6.5 ± 2.3
Alpha (degree)	23.0 ± 5.9	<0.001 ns	44.3 ± 17.2# 33.1 ± 13.3
MA (mm)	42.6 ± 5.4	<0.001 <0.001	62.1 ± 8.4 57.5 ± 9.1
Trombo (x10^9/l)	245 ± 48	ns ns	259 ± 64 229 ± 70
PT (sec)	12.6 ± 0.7	<0.001 <0.001	14.4 ± 0.7 14.9 ± 0.7
APTT (sec)	32.0 ± 3.2	ns ns	30.4 ± 2.6 30.3 ± 2.9
Fibrinogen (g/l)	2.7 ± 0.5	0.04 <0.001	3.1 ± 0.9 3.7 ± 1.1
AT (%)	105 ± 10	ns ns	101 ± 10 97 ± 31

* Displayed values are mean ± standard deviation. # p<0.05 HU- vs. HU+
SCD: Sickle cell disease HU -; Hydroxyurea non-user, HU +; Hydroxyurea user.
R-time; reaction time, K-time; clotting time, Alpha; alpha angle, MA; maximum amplitude.
Trombo; trombocytes, PT; prothrombin time, APTT; activated partial tromboplastin time, AT; antithrombin, ns; not significant.

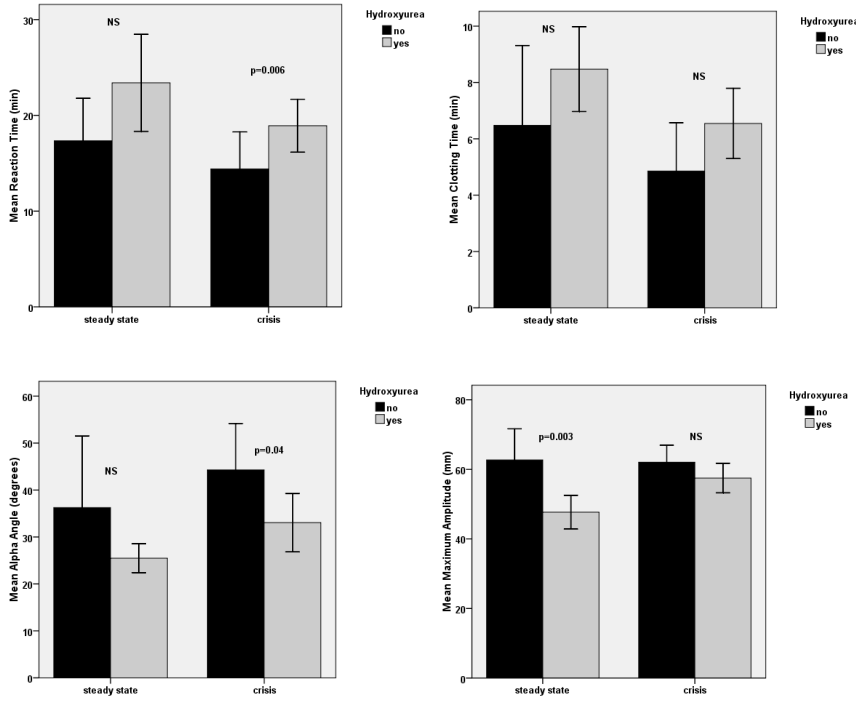


Figure 1.

Bar charts (+95%CI) representing TEG parameters in Hydroxyurea users- and non-users, in steady state and in crisis

Association of TEG variables with parameters of SCD activity

A lowered Hb and increased leucocyte number are considered part of disease activity. We observed statistically significant correlations between most TEG parameters and both Hb level and leucocyte count, suggesting an enhancement of the clotting process in patients with leucocytosis and/or progressive anaemia. The scatter diagrams and regression equations of TEG parameters versus Hb level respectively leucocyte count are presented in Figures 2 and 3. Decreasing Hb level was associated with a shortening of the rate of initial fibrin formation (R-time; r = 0.43) and with the time until a fixed level of clot firmness was reached (K-time; r = 0.45). Moreover, lower Hb levels were associated to an increase in both clot growth (alpha angle;

r = -0.58) and the maximum strength or stiffness of the developed clot (MA; *r* = -0.72). A higher leucocyte count was also associated with both an increase in the rate of fibrin formation (R-time; *r* = -0.31 and K-time; *r* = -0.24) and with a faster clot growth (alpha angle; *r* = 0.41) and improved ultimate clot strength (MA; *r* = 0.41). Remarkably, no correlations were found between Platelet count and any of the TEG parameters. Further we found that the level of Fetal Hemoglobin (HbF) was positive correlated to the rate of initial fibrin formation (R-time; *r* = 0.43) and negative to the ultimate clot strength and stiffness (MA; *r* = -0.41).

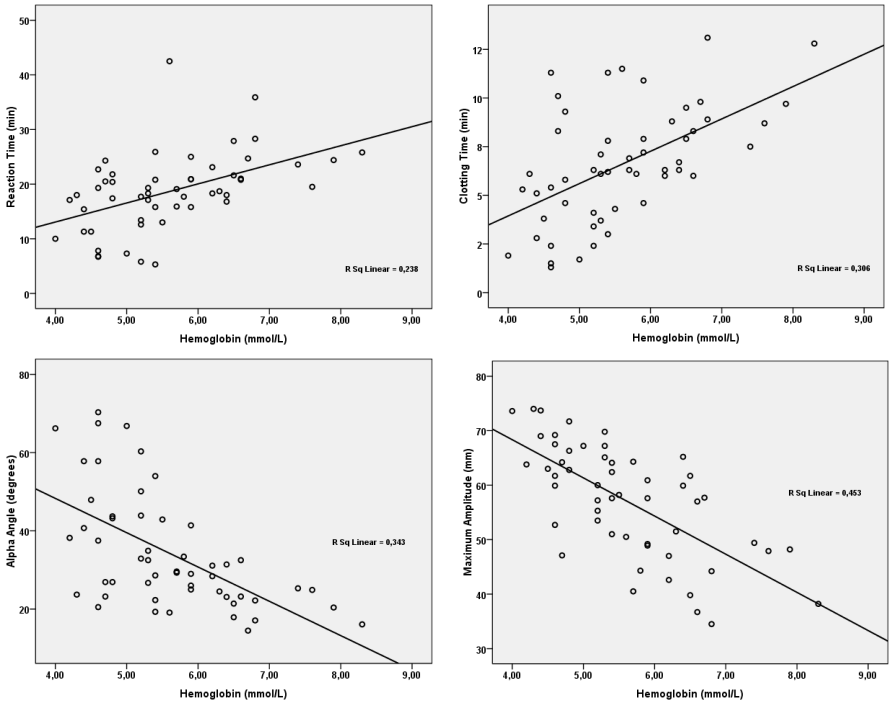
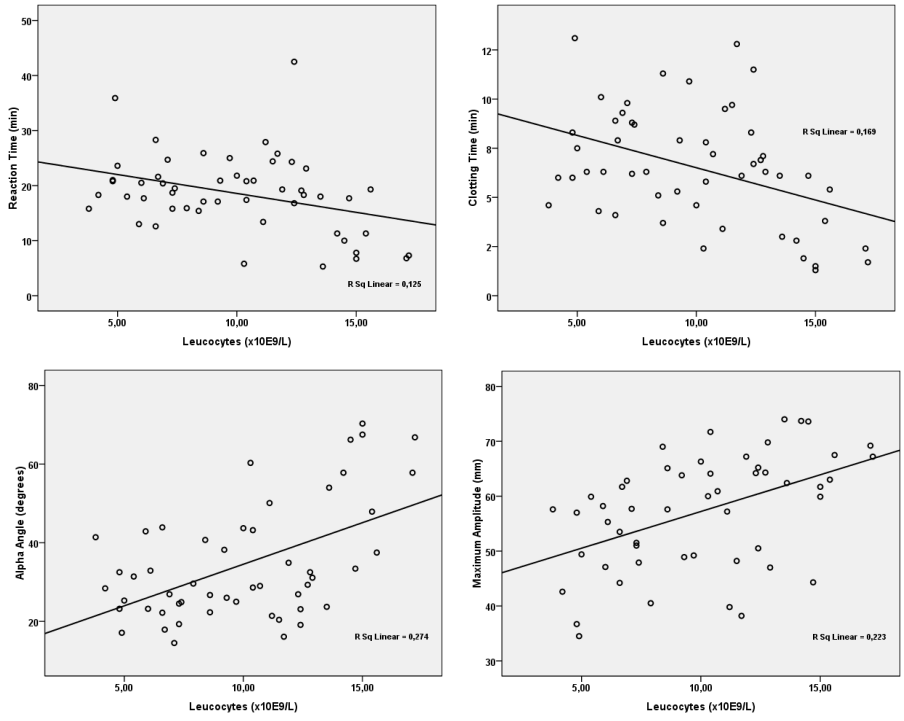


Figure 2.

Correlation and linear regression of Hemoglobin values with various TEG parameters. (R Sq linear = correlation coefficient)



Figures 3.

Correlation and linear regression of Leucocyte values with various TEG parameters. (R Sq linear = correlation coefficient).

DISCUSSION

The procoagulant profile observed in patients with SCD is complex and multifactorial. Instead of analyzing all the separate underlying haemostatic aspects with different tests, the thrombelastography technique has the advantage of integrating all these (dynamic) aspects of coagulation into one single test. Apart from a study in pediatric patients by Yee and colleagues (21), we are the first to report on TEG variables observed in patients with SCD, both in steady state and in crisis.

In the current study in adult patients with SCD, not using HU, we observed hypercoagulable TEG parameters, both in steady state and during crisis, when compared with healthy age matched controls. This procoagulant TEG profile involved both the initiation and propagation of thrombin formation (respectively Reaction-time, Clotting-time and Alpha angle), but also the ultimate clot strength and clot elasticity (Maximum Amplitude). Classical coagulation tests demonstrated higher platelet- and fibrinogen- levels as well as shortened APTT in patients with SCD in steady state compared to controls. Moderate thrombocytosis is characteristic of patients with SCD and has been attributed to functional asplenia and the lack of splenic sequestration (22).

Although TEG parameter Maximum Amplitude is propagated to reflect both plateletcount and platelet activity as well as fibrinogen level, we did not observe a significant correlation between MA and these two classical coagulation test. A possible explanation for this finding is the relatively small distribution wide of both platelet counts and fibrinogen levels in our study group.

Our findings are in accordance with those of Yee and colleagues who observed hypercoagulable TEG patterns in pediatric patients with SCD. However, in their study recalcified citrated whole blood samples were used, instead of “unmanipulated” fresh whole blood as we did. TEG with recalcified citrated blood is used as an alternative to non-citrated blood in situations in which immediate TEG determination is not practically feasible. However, the use of a citrated sample generates different results than those obtained with fresh whole blood making both techniques not interchangeable (20,23).

During crisis, TEG profiles of patients with SCD became even more pro-coagulant compared to the profiles observed in steady state. This procoagulant state was observed for all measured TEG parameters, but not for the classical coagulation tests. The significant lower platelet count is probably caused by decreases in lifespan accompanying vaso-occlusive crisis. Demonstration of a procoagulant state during a vaso-occlusive crisis is (indirectly) supported by other studies in which during crisis

an increase in the levels of markers of thrombin generation as well as an increase in the expression of tissue factor was observed (6,24).

To date, the only agent that has been specifically approved for the treatment of SCD is HU, a drug known to increase HbF and to reduce white blood cell count. HU has been shown to decrease the frequency of painful crises, episodes of acute chest syndrome, hospitalizations, and blood transfusions (6,25). Our data clearly illustrate the effects HU has on the coagulation profile in patients with SCD as measured by TEG. When HU users and HU naïve patients were compared to controls, the HU naïve group had significant procoagulant TEG parameters compared to controls, whereas HU users only had significant elevated Maximum Amplitude. This suggests that HU particularly has (beneficial) influence on the initiation and propagation phase of the coagulation profile, but less on clot strength and clot elasticity. Unexpectedly, however not statistically significant, we observed higher platelet counts in the HU treatment group compared to the HU naïve group.

A high level of HbF in SCD correlates with a decrease in the incidence of vaso-occlusive crises (26). The latter is in agreement with our observation of significant correlations of HbF level with both Reaction Time (r =0.43; $p<0.01$) and Maximum Amplitude (r =-0.41; $p<0.05$). In terms of coagulation; with the increase in HbF level (as a result of HU treatment), both a delay in the initiation phase of coagulation as well as a decrease in ultimate clot strength and clot elasticity were observed.

In this study we demonstrated that red and white blood cells do play a role in haemostasis in patients with SCD as both were significantly correlated to TEG parameters corresponding to different parts of the coagulation cascade. In case of increasing Hb level, we observed a significant delay in the initiation phase of plasmatic coagulation (as was demonstrated by the finding of an increase in both Reaction-Time and Clotting-Time (r = 0.43 and r = 0.45; $p<0.01$) and a decrease in cloth growth and ultimate clot strength, corresponding to a decrease in both Alpha Angle and Maximum Amplitude (r = -0.58 and r = -0.77; $p<0.01$). These findings suggest that progressive anaemia in patients with SCD is accompanied by a hypercoaguble state.

The latter is (partly) in accordance to observations made in an earlier study performed in (healthy) patients with anaemia in which lower Hb levels were associated with an increase in ultimate clot strength and elasticity (27). However in that study, increasing Hb level was correlated to a faster initiation of plasmatic coagulation.

Further we observed that leucocytosis in patients with SCD led towards a hypercoagulable TEG profile, with both a faster initiation and propagation phase of coagulation (K-time; r = -0.31; $p<0.05$ and alpha angle; r = 0.41; $p<0.01$) and an increase in clot strength and clot elasticity (MA; r = 0.41; $p<0.01$). We assume that lower Hb levels and higher leucocyte counts in patients with SCD are representatives of an increased in vivo sickling activity, resulting in a procoagulant haemostatic profile. An important limitation of our study is that we did not match our controls for ethnicity, which may have implications for the interpretation of the results. Hagger et al observed in their study significant differences in parameters of coagulation between Black and Caucasian control subjects (28). Moreover, in clinical practice, where TEG is used as a rapid point-of-care-test of haemostasis, coagulation activators are often added to the blood samples. As we used only whole blood samples without activators, the study observations may not be applicable to other modalities of TEG measurement. Finally, despite the evidence that the coagulation system is activated in patients with SCD, we did not prove that this procoagulant state is etiologically linked to the vascular occlusion and end-organ damage that is characteristic of SCD. However, we think the coagulation profile as determined by TEG might be of value in both predicting clinical behavior and in diagnosing (the severity of) a painful crisis. Moreover TEG could be used as a tool to monitor the effects treatment with HU has on the coagulation profile. In order to further elucidate the role of TEG in SCD, further prospective studies are warranted with focus on clinical course- and outcome, in relation to TEG profiles, in individual patients with SCD.

In summary, in patients with SCD we observed a procoagulant TEG profile compared to matched healthy controls. Moreover, vaso-occlusive crisis led to a further increase of this procoagulant state, with beneficial effects of HU treatment on this pattern. Finally, we demonstrated that anaemia and leucocytosis in SCD are both linked to

hypercoagulability. As the pathogenesis of vaso-occlusion in SCD is multifactorial, the exact contribution of hypercoagulability remains to be determined. It is uncertain whether the haemostatic changes observed are causative of disease specific complications or are simple epiphenomena. As a result, further studies are required to extend and confirm the observed hypercoagulable state in patients with SCD and the potential beneficial effects of HU.

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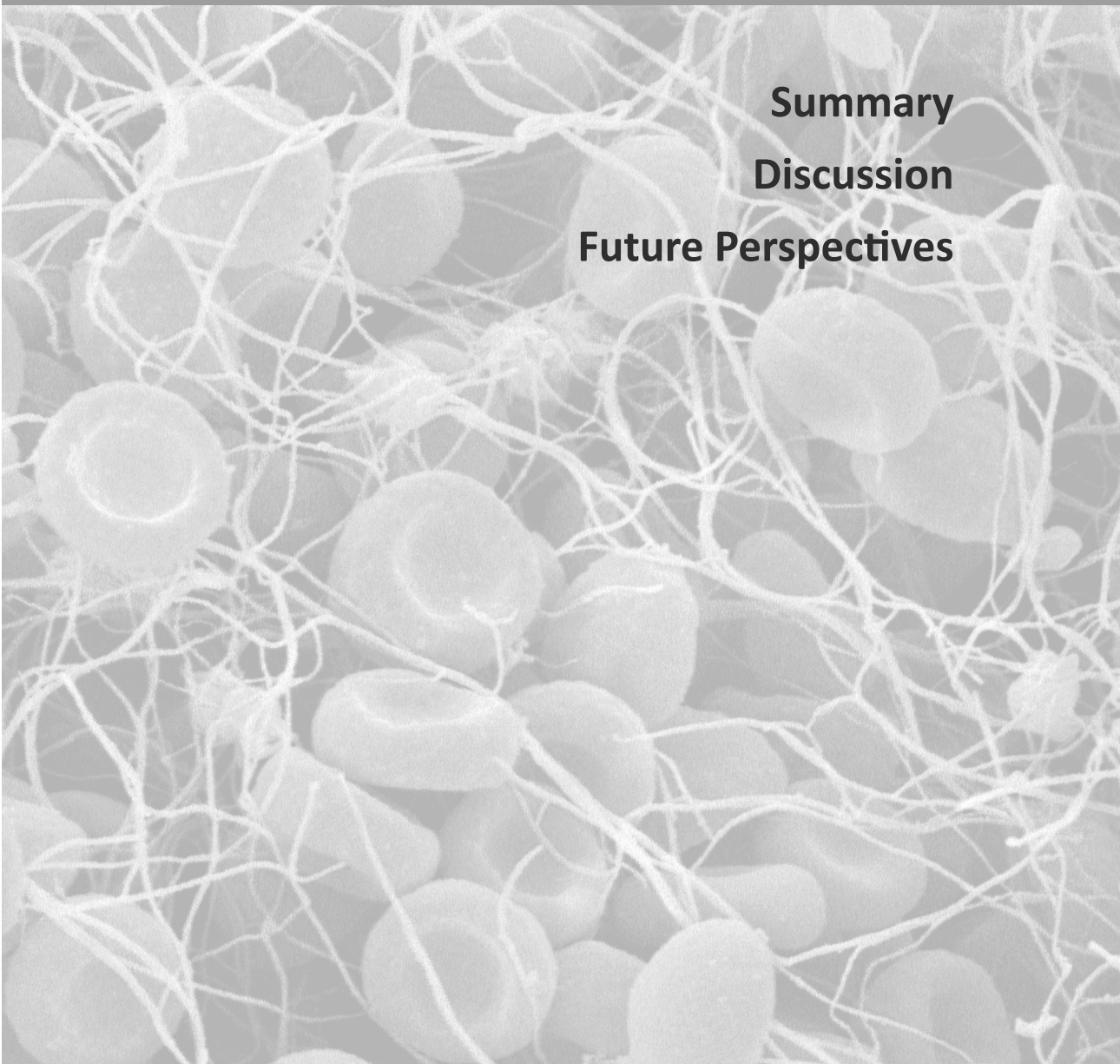

Summary

Discussion

Future Perspectives

SUMMARY

Laboratory evaluation of haemostasis has been performed using standard clotting assays for several decades. The cell-based model of coagulation has led to renewed interest in Thromboelastography (TEG), as this technique has the potential for allowing a new look at the process of haemostasis, which is of interest for both clinicians and researchers in the field of haemostasis (1-3). TEG is a global test of haemostasis that visualizes the viscoelastic changes that occur during coagulation *in vitro*. The technique provides a graphic representation of the quality of the clot and the dynamics of its formation and subsequent lysis. TEG has the advantage over classical coagulation tests that it is performed bedside and in whole blood, offering a rapid overview of the sum of the cumulative effects of platelets and plasma factors as well as interacting cellular elements. In contrast, classical coagulation tests are performed in plasma under conditioned circumstances and give fragmented information on the initiation of the coagulation cascade, and are especially useful in analyzing isolated defects in this cascade. TEG was initially used to guide transfusion in the setting of hepatic-, cardiovascular-, and trauma surgery where haemostatic disturbances are complex and multifactorial (4-7). Moreover, when incorporated into a transfusion algorithm, TEG might add to the management of massive blood loss (8,9). Nowadays, TEG is used by a growing number of clinicians and its use has been expanded to all other areas of haemostasis- and thrombosis-testing.
The scope of this thesis has been to improve the understanding of TEG technology for clinicians working in the field of haemostasis, making them aware of the possibilities, but also of the limitations of TEG in coagulation monitoring and haemostasis research.

In *Chapter 2* an overview was given on the role of TEG and other point-of-care (POC) tests of haemostasis in both the prediction and treatment of massive blood loss. For this purpose the current (cell-based) model of haemostasis was lined out with special focus on the coagulopathy seen in massive blood loss. Further, we discussed the characteristics of the “ideal” POC test of haemostasis. After describing classical coagulation tests we focussed on the different POC analysers and methods available and gave insight into which coagulation parameter(s) can be measured and whether these tests have clinical benefit in the management of massive blood loss.

In *Chapter 3* we described the effects of age, gender and the use of oral contraceptives on coagulation. TEG parameters were measured in both native and citrated whole blood from 120 healthy adults (60 men, 60 women) at various ages, and in an additional 29 healthy women using oral contraceptives. We observed hypercoagulabiltiy in females compared to males and in women using oral contraceptives compared to age-matched non-users. Moreover, we observed hypercoagulability with aging. Using the method of Bland Altman, we demonstrated no correlation between TEG measurements in native and recalcified citrated blood. We concluded that aging, female gender, use of oral contraceptives and low-normal haematocrit levels have significant procoagulant effects. TEG measurements in native and recalcified citrated blood are not interchangeable as our data showed a lack of correlation between the TEG measurements in both types of blood samples, due to the very large limits of agreement.

In *Chapter 4* we analyzed the influence of platelet count on coagulation. We performed TEG analyses serially in patients with well documented transient thrombocytopenia. A total of 189 TEG analyses were performed in 16 patients with a hematological malignancy, all in remission, receiving consolidation courses of chemotherapy. TEG outcome using native and citrated blood samples at a median of 11 times (range 1-17) in the same patients during the decrease of platelet count in response to chemotherapy were compared to outcome in 120 healthy adults from various age categories. We found an excellent correlation (r = 0.7; p<0.001) between TEG clot strength (maximum amplitude) and platelet count. Moreover, platelet count was correlated with respectively the initial rate of clot formation (reaction time and clotting time), the rate of clot growth (alpha angle), and also with maximum thrombus generation, time to maximum thrombus generation and total thrombus generation. We concluded that platelet count not only affects the strength of clot formation, as was expected, but also all other phases of plasmatic coagulation. Citration of the blood sample, aiming at easy storage of the material, masked some of the important biological parameters of coagulation.

In *Chapter 5* we described the haemostatic function of both transfused platelets, in relation to native circulating platelets. Further we reported on the effect of storage time of platelet concentrates on their haemostatic potential. During the decrease in platelet count after chemotherapy, TEG parameters were measured serially until the transfusion trigger was reached in 92 patients. TEG parameters for different ranges of native circulating platelets could be assessed, which were compared to ranges obtained in the thrombocytopenic period in which the patient received platelet transfusions. Finally we compared the haemostatic potential of fresh platelet concentrates (1-3 days) with platelet concentrates with longer storage time (4-5 days). We found no differences in haemostatic potential between native platelets and transfused stored platelets (all *p* values ≥0.1). The transfusion of fresh platelets demonstrated better haemostatic effects than longer stored platelets. Both the time until a fixed level of clot firmness was reached (K-time) as well as the rate of clot growth (alpha angle) were superior for fresh platelet concentrates. We concluded that TEG could monitor the haemostatic effects of platelet transfusion, with comparable haemostatic properties of native circulating platelets and transfused stored platelets. Further, our data suggested that limited storage time is associated with a better haemostatic capacity.

In *Chapter 6* we conducted a study with TEG on the role of red blood cells (RBCs) in haemostasis. In 29 patients with chemotherapy induced anaemia we studied the effect of progressive anaemia on the coagulation profile. In 24 patients with chronic anaemia we studied the effect of transfusion of RBCs on coagulation. Finally, we evaluated in 18 patients whether storage time of RBCs had additional effects on haemostasis. We observed a significant negative correlation between haemoglobin and TEG variables related to both cloth strength and elasticity ($p<0.05$). Moreover, anaemia was associated with a delay in the initiation of the coagulation cascade. Correction of anaemia by RBC transfusion resulted in significant shortening of this initiation phase with now the opposite effect on clot strength and elasticity. The negative effects on clot quality were significantly worse when fresh RBCs were transfused compared to longer stored RBCs. Furthermore, in contrast to the longer stored RBCs, fresh RBCs did not enhance initial fibrin formation. We concluded that

anaemia is associated with a delay in the initiation of the coagulation cascade with a finally formed clot with superior strength and visco-elastic properties. Transfusion of RBCs is associated with impaired clot quality, with even worse effects on the initial fibrin built up and clot quality by fresh RBCs.

In *Chapter 7* we described the use of TEG in analysing hypercoagulability in patients with sickle cell disease (SCD), both in steady state and in painful crisis. Further, we described the effects of Hydroxyurea (HU) and the role of red and white blood cells on haemostasis in SCD. In 18 patients with SCD (11 on HU), coagulation parameters could be measured in steady state and during 34 painful crises. Coagulation parameters in SCD patients were compared to controls and steady state was compared to crisis, in relation to HU use. We observed statistically significant procoagulant TEG parameters in the SCD population not using HU compared to the control group both in steady state and in crisis. Apart from the ultimate clot strength, HU significantly reduced hypercoagulability compared to both controls and HU non-users. Further, both red and white blood cells were respectibely negatively and positively correlated to hypercoagulability. We concluded that in patients with SCD, TEG might be a useful tool to both identify and monitor hypercoagulability as well as to detect the effects of treatment with HU.

DISCUSSION AND FUTURE PERSPECTIVES

In recent years a growing number of articles and studies on TEG have been published, covering different areas of clinical medicine. Despite these publications, TEG has been considered a research tool for a long time, and the technique has been criticized because of the lack of studies demonstrating its reliability. Indeed, the methodology has drawbacks concerning validation and standardisation. The latter results into significant intra- and inter-laboratory variability, making comparison between results difficult (10-12). Further, studies comparing TEG parameters with standard coagulation tests demonstrate poor correlation and there are no clinical trials that link TEG variables to clinical outcome. Moreover, randomized studies evaluating TEG-guided transfusion therapy versus fixed ratios of blood components

in patients with massive blood loss are missing. Therefore, implementation of such an algorithm in the management of massive blood loss seems preliminary as discussed in *Chapter 2*. A Cochrane review published in 2011 also concluded that there is an absence of evidence that the use of TEG or ROTEM improves morbidity or mortality in patients with severe bleeding. Application of a TEG or ROTEM guided transfusion strategy seemed to reduce the amount of bleeding, but failed to show any statistically significant effect on other predefined outcomes as the incidence of surgical reoperation due to bleeding, mean length of stay in the intensive care unit and number of days in the hospital (13).

In an effort to improve reproducibility and to standardize TEG, an international group of investigators joined hands in the year 2010 to form the TEG-ROTEM Working Group (14). In their first study a significant inter-laboratory variance with coefficients of variation greater than 10% was demonstrated (15). Their study has been the first effort to standardize TEG methodology. It shows that still significant work remains to be done to improve reliability and reproducibility. In line with the Working Group's call for additional research on validation of TEG, our study group assessed own reference values in a large random study population as presented in *Chapter 3*. So far we are the first to study a population with a well-balanced age and gender distribution and describe both classical and dynamic TEG variables and compare these with classical coagulation tests, both in native and in citrated whole blood. Although impractical, our study underlines the manufacturer's recommendation that each institution should determine its own normal values before adopting TEG. Moreover, it is important to emphasize the fact that TEG measurements in native and citrated blood are not interchangeable. Further we found clear procoagulant effects of age, gender and use of oral contraceptives on haemostasis, underlining the sensitivity of TEG over classical coagulation tests in detecting these differences in the haemostatic profile. Although the observed procoagulant effects of aging and female gender were subtle and probably physiological, clinicians and researchers working with TEG should be aware of these effects when interpretating test results.

In the operating theatre TEG is increasingly used to guide transfusion- and haemostatic- therapy as the technique offers the practical advantage of real-time coagulation monitoring with subsequent treatment, based on TEG profile and TEG parameters. Low platelet counts (<50 x 10^9/L) are considered a major risk factor for bleeding complications (16). Although TEG parameter Maximum Amplitude (MA) is classically (mainly) influenced by platelet count, we found this parameter to be insensitive in detecting low platelet counts, as we describe in Chapter 4 (17,18). Importantly, when platelet count dropped below 50 x 10^9/L, TEG performed with native whole blood showed a flat TEG curve in over more than 40% of the analyses. Further we observed that thrombocytopenia affected all other TEG parameters, which makes TEG based haemostatic therapy hazardous in these clinical situations, at least when non activated blood samples are used. Moreover, for proper use and interpretation of the TEG assay, it is important that also the effects of other isolated coagulation defects as well as the effects of haemostatic agents are evaluated and validated, before a TEG guided transfusion algorithm can be implemented to manage hemorrhage.

Platelet transfusion is used therapeutically in patients with quantitative or qualitative platelet disorders who are actively bleeding, or as prophylaxis in patients who are at serious risk of bleeding (19). Optimally, transfused platelets should have the same haemostatic capacity as native circulating platelets. Although *in vitro* tests and markers are performed on the platelet product, these tests do not provide information on the haemostatic *in vivo* potential. In Chapter 5 we describe how TEG can monitor the haemostatic effects of platelet transfusion, with comparable haemostatic properties of native circulating and transfused stored platelets. Further, our data suggest that limited storage time is associated with a better haemostatic capacity, at least during the initial 24 hours after transfusion. The latter might have clinical implications in terms of transfusing only fresh platelet products to patients with active bleeding, whereas stored platelet products can be reserved when platelets are transfused for prophylaxis. However, a change in platelet transfusion policy is preliminary, as we did not measure the (clinical) effects of platelet transfusion on bleeding episodes. Future studies relating haemostatic capacity of fresh and stored platelet products (measured by TEG) to bleeding episodes are therefore necessary.

Although the primary goal of RBC transfusion is to improve oxygen delivery, RBCs also have effects on haemostasis as described in Chapter 6 and 7. We found anaemia to be associated with a delay in clot initiation with a finally formed clot of superior quality. Interestingly, transfusion of RBCs was accompanied by exactly the opposite effects on haemostasis, with faster clot initiation at the cost of clot quality. Remarkably, RBCs with a storage time of only 2 days did not demonstrate this procoagulant effect on clot initiation; clot quality deteriorated, however. Both observed phenomena can probably be explained. First, RBCs positively influence the initiation of haemostasis, as under flow conditions they move toward the centre of the vessel, causing a near wall excess of platelets with subsequent adhesion, activation and aggregation of these platelets. Second, the RBC-inhibiting effect on TEG clot strength is probably caused by the formation of a looser clot structure with increasing amounts of RBC built in the fibrinogen network. Recently, our results were confirmed by an *in vitro* study where increasing amounts of RBC also reduced clot strength (20). We consider our observation of importance for workers in the field of haemostasis using TEG, as they should be aware of the effects of haematocrit on haemostasis when analyzing TEG parameters. Further, we can only speculate whether the haemostatic effects of RBCs are somehow related to the worse clinical outcome as seen in critically ill patients, in relation to the number of received RBC transfusions (21). The importance of RBC in haemostasis is also highlighted in Chapter 8, where we used TEG to study haemostasis in patients with sickle cell disease (SCD). Although the procoagulant state observed in these patients is considered to be multifactorial, there is no single test to quantify or qualify this condition. With TEG we could visualize and measure this procoagulant state, which was even more pronounced during painful sickling crisis. Moreover, we could also detect the beneficial effects of treatment with Hydroxyurea on this procoagulant state. It remains however uncertain whether the haemostatic changes observed are causative of disease specific complications or are simple epiphenomena. As a consequence, further studies are required to relate TEG parameters to clinical outcome, in relation to HU use.

We consider TEG a promising tool for the evaluation of haemostasis, both in the clinical and experimental field, because the technique allows a unique, more physiological view at haemostasis. In contrast to classical coagulation tests, and in spite of it's global character, the technique can detect age, gender and hormonal effects on the coagulation profile. Furthermore, TEG seems capable in demonstrating the effects of platelets and red blood cells on haemostasis and has potential as an *in vivo* quality test of blood products. Also, TEG might play a future role in monitoring and treating patients with SCD. However, despite it's enormous opportunities, the TEG technique still has several limitations. Although in recent years much progress has been made to improve its reliability and reproducibility as well as to standardize and validate the different test modifications and applications, the technique still cannot be utilized to its full capacity, mainly because of inconsistencies in study results. Importantly, benefits to the patient need to be well documented to enable a more widespread use of TEG haemostasis testing in the operating theatre. Moreover, we want to emphasise the importance of obtaining own reference ranges for comparison. Further, as far as quality assurance is concerned, it is no less important than for conventional laboratory-based coagulation tests to deploy both internal quality control and external quality assessment. We hope that much more investment is directed to TEG studies in both experimental and clinical fields to improve applications and promote use. Targeted use of TEG, interpretation of the curves based on the clinical situation and combined with information from classical coagulation tests, may be beneficial for the future patients.

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9

Nederlandse samenvatting
Discussie
Toekomstperspectieven

NEDERLANDSE SAMENVATTING

Bij de analyse van de stollingsstatus van een patiënt wordt sedert decennia gebruik gemaakt van klassieke stollingstesten. Het huidige cellulaire model van stolling, met meer aandacht voor de rol van rode en witte bloedcellen alsmede bloedplaatjes, heeft aanleiding gegeven tot hernieuwde interesse in de Tromboelastografie (TEG). Met de TEG techniek wordt op een unieke en meer fysiologische manier naar stolling gekeken, wat interessant is voor zowel clinici als onderzoekers actief op het gebied van hemostase. TEG is een globale hemostasetest die *in vitro* de visco-elastische veranderingen van de stolselvorming visualiseert. De techniek levert een grafische weergave van niet alleen de vorming en afbraak van het stolsel, maar ook van de kwaliteit van het stolsel. Het voordeel van TEG is dat het onderzoek direct aan het bed van de patiënt in volbloed kan worden verricht. De test geeft zo een snel overzicht van de cumulatieve hemostatische effecten van bloedplaatjes, plasmafactoren en cellulaire bloedelementen. Klassieke stollingstesten daarentegen worden onder geconditioneerde omstandigheden in plasma uitgevoerd en geven gefragmenteerde informatie over met name de initiatie van de stollingscascade. Klassieke stollingstesten zijn derhalve met name informatief bij de analyse van geïsoleerde stollingsdefecten. TEG vindt historisch zijn toepassing in leverchirurgie, in cardiovasculaire chirurgie en bij traumata, waarbij de optredende stollingsstoornissen vaak complex en multifactorieel van aard zijn. Daarnaast kan TEG van nut zijn bij de behandeling van patiënten met massaal bloedverlies wanneer deze test wordt geïncorporeerd in een transfusiealgoritme. Tegenwoordig is TEG een test die snel aan populariteit wint bij uiteenlopende disciplines en die wordt ingezet in uiteenlopende deelgebieden van stollings- en tromboseonderzoek.

Het belangrijkste doel van dit proefschrift was om het kennisniveau van clinici die gebruik maken van de TEG te verbeteren, waarbij het van belang is dat de gebruiker niet alleen op de hoogte is van de mogelijkheden, maar ook van de potentiële beperkingen en bezwaren die deze test met zich meebrengt.

In *Hoofdstuk 2* is een overzicht gegeven van de rol die TEG en andere zogenaamde "point-of-care" (POC) hemostasetesten kunnen spelen in het voorspellen en behandelen van massaal (operatief) bloedverlies. Allereerst werd om deze reden het huidige cellulaire hemostase model uiteengezet, met extra aandacht voor de specifieke stollingsstoornissen zoals deze optreden tijdens massaal bloedverlies. Daarnaast zijn de karakteristieken beschreven waaraan de "ideale" POC hemostasetest zou moeten voldoen. Nadat inzicht is gegeven in de achtergrond van de klassieke stollingstesten, hebben wij ons gericht op de diverse POC technieken en testen. Tevens hebben wij onderzocht welke stollingsparameters hiermee specifiek kunnen worden bepaald en of de test potentiële klinische waarde heeft bij massaal bloedverlies.

In *hoofdstuk 3* zijn de effecten van leeftijd, geslacht en gebruik van orale anticonceptiva (OAC) op de stolling beschreven. TEG parameters werden bepaald in volbloed en in citraat-ontstold volbloed in 120 volwassenen (60 vrouwen, 60 mannen) met variabele leeftijdsopbouw en in een extra groep van 29 jonge vrouwen die OAC gebruikten. Een toename in neiging om te stollen werd aangetroffen bij vrouwen ten opzichte van mannen, bij OAC gebruiksters ten opzichte van jonge vrouwen die geen OAC gebruikten en bij vorderende leeftijd. Daarnaast vonden we geen correlatie tussen TEG parameters bepaald in volbloed en TEG parameters bepaald in citraat-ontstold bloed volgens de methode van Bland Altman. We concludeerden dat leeftijd, vrouwelijk geslacht, OAC gebruik en laag normale hematocrietwaarden aanleiding geven tot een significante toename in de neiging om te gaan stollen. Daarnaast kunnen TEG parameters bepaald in volbloed niet uitgewisseld worden met die van citraat-ontstold volbloed aangezien er onvoldoende mate van overeenkomst is tussen beide testmethoden.

In *hoofdstuk 4* analyseerden we het effect dat het bloedplaatjesaantal heeft op de stolling. Hiervoor verrichtten we een serie TEG metingen bij patiënten met goed gedocumenteerde passagère trombocytopenie. In totaal werden 189 TEG metingen verricht in 16 patiënten, allen bekend met een hematologische maligniteit in remissie, die behandeld werden met consoliderende chemotherapiekuren. Gemiddeld werden per patiënt 11 TEG metingen in volbloed en citraat-ontstold volbloed

verricht (range 1-17) tijdens het dalen van het bloedplaatjesaantal. De uitkomsten werden vergeleken met die van 120 gezonde proefpersonen met variabele leeftijd. We vonden een uitstekende correlatie ($r = 0.7$, $p < 0.001$) tussen de sterkte van het stolsel (maximale amplitude) en het bloedplaatjesgetal. Daarnaast was het bloedplaatjesgetal gecorreleerd met de initiële snelheid van stolselvorming (R-tijd en K-tijd), de snelheid van de groei van het stolsel (alphahoek) alsook met de nieuwere TEG parameters (MTG, TMG, TTG). We concludeerden dat het bloedplaatjesaantal niet alleen effect heeft op de elastische trekkracht van het gevormde stolsel, maar ook op alle andere fases van de plasmatische stolling. Ontstollen van volbloed middels citraat maskeerde overigens een aantal belangrijke biologische parameters van stolling.

In *hoofdstuk 5* is de hemostatische functie van getransfundeerde bloedplaatjes vergeleken met die van autologe (natieve) circulerende bloedplaatjes. Tevens werd het effect van de opslagduur van deze bloedplaatjes op hun hemostatische functie bestudeerd, waarbij verse bloedplaatjes (opslagduur 1-3 dagen) werden vergeleken met langer opgeslagen bloedplaatjes (opslagduur 4-5 dagen). In 92 patiënten, behandeld met chemotherapie, zijn tijdens de daling van het bloedplaatjesaantal op meerdere momenten TEG analyses uitgevoerd totdat de patiënt afhankelijk werd van een bloedplaatjestransfusie. TEG parameters voor verschillende ranges van natieve bloedplaatjesaantallen werden vergeleken met diezelfde ranges verkregen tijdens een bloedplaatjestransfusie. Er werd geen verschil aangetoond tussen de hemostatische functie van autologe en getransfundeerde bloedplaatjes (alle p waarden ≥ 0.1). De hemostatische effecten van verse bloedplaatjes waren overigens beter dan die van opgeslagen bloedplaatjes voor wat betreft de tijd die nodig was om een kritische hoeveelheid stolsel te produceren (K-tijd) alsook de snelheid waarmee het stolsel werd gevormd (alphahoek). We concludeerden in deze studie dat TEG in staat was om de hemostatische effecten van een bloedplaatjestransfusie te monitoren, waarbij de functie van getransfundeerde bloedplaatjes vergelijkbaar was met die van natieve bloedplaatjes. Daarnaast suggereerden onze data dat kortere opslagduur van bloedplaatjes geassocieerd was met een betere hemostatische capaciteit.

In *hoofdstuk 6* is de rol van rode bloedcellen (RBC) in de hemostase bestudeerd middels TEG. Allereerst werd in 29 met chemotherapie behandelde anemische patiënten het effect van een dalend hemoglobine getal op de stolling beschreven. Vervolgens werd in 24 patiënten met chronische anemie het effect van een transfusie met RBC op de stolling beschreven. In nog eens 18 patiënten werd bestudeerd of de duur van opslag van rode bloedcelconcentraten van additionele invloed was op deze hemostatische effecten. Wij vonden een significant negatieve correlatie tussen hemoglobine en de TEG variabelen corresponderend met de elastische trekkracht van het gevormde stolsel ($p < 0.05$). Daarnaast was anemie geassocieerd met het trager op gang komen van de stollingscascade. Het transfunderen met RBC in geval van anemie daarentegen leverde exact de tegenovergestelde hemostatische effecten op met nu een verslechtering van de uiteindelijke elastische trekkracht van het stolsel waarbij de stollingscascade wel sneller op gang kwam. De genoemde negatieve effecten op de elastische kwaliteit van het stolsel werd met name gezien wanneer verse rode bloedcelconcentraten werden gegeven. Verse rode bloedcelconcentraten gaven overigens geen versnelling van het begin van de stollingscascade. De conclusie van deze studie was dat anemie enerzijds geassocieerd is met een vertraging van de aanvang van de stollingscascade en anderzijds met een verbetering van de elastische kwaliteit van het gevormde stolsel. Transfusie van met name verse RBC is geassocieerd met verslechtering van de elastische kwaliteit van het gevormde stolsel.

In *hoofdstuk 7* is de rol van TEG beschreven bij het analyseren van de verhoogde stollingsstatus bij patiënten met sikkelcelziekte, zowel tijdens pijnlijke vaso-occlusieve crises als ook tijdens klinisch rustige perioden. Daarnaast zijn de effecten van behandeling met Hydroxyureum (HU) alsook de rol van rode en witte bloedcellen op hemostase bij deze aandoening bestudeerd. Bij 18 patiënten met sikkelcelziekte (waarvan 11 HU gebruikten) werden stollingsparameters in rust bepaald en tijdens in totaal 34 pijnlijke crises. Stollingsparameters van patiënten met sikkelcelziekte werden vergeleken met die van gezonde controlepersonen en de stollingsstatus in rust werd vergeleken met die tijdens pijnlijke crises in relatie tot gebruik van HU. Wij observeerden bij patiënten met sikkelcelziekte zonder HU gebruik een significante toename in stollingsstatus in vergelijking met controle personen, zowel tijdens crises

als in rust. Met uitzondering van de uiteindelijke trekkracht van het gevormde stolsel, verminderde HU gebruik deze toegenomen stollingsstatus significant in vergelijking met zowel patiënten die geen HU gebruikten als met controlepersonen. Rode en witte bloedcellen waren tenslotte respectievelijk negatief en positief gecorreleerd met een toegenomen stollingsstatus. De conclusie was dat TEG een potentieel interessante techniek is om de stollingsstatus bij patiënten met sikkelcelziekte te analyseren en te vervolgen, waarbij ook de effecten van behandeling met HU kunnen worden gedetecteerd.

DISCUSSIE EN TOEKOMSTPERSPECTIEVEN

In de afgelopen jaren zijn een groeiend aantal artikelen en studies over TEG gepubliceerd die vrijwel alle deelgebieden van de geneeskunde omvatten. Ondanks deze publicaties werd TEG nog lange tijd als experimenteel beschouwd en bekritiseerd vanwege een gebrek aan studies die de betrouwbaarheid van deze techniek aantonen. Een belangrijk nadeel is de beperkte validatie en standaardisatie van de TEG methodiek, resulterend in aanzienlijke variatie in uitkomsten, zowel tussen laboratoria alsook binnen één laboratorium, wat onderlinge vergelijking van uitkomsten problematisch maakt. Bovendien laten vergelijkende studies slechts zeer beperkte correlaties zien tussen TEG parameters en klassieke stollingstesten. Tenslotte zijn er vrijwel geen studies verricht waarbij TEG werd gerelateerd aan de klinische uitkomst. Belangrijk is dat goede gerandomiseerde studies ontbreken die een op TEG gebaseerd transfusiealgoritme vergelijken met een gefixeerd en op klassieke stollingstesten gebaseerd transfusiealgoritme in geval van massaal bloedverlies. Om deze redenen lijkt het vooralsnog prematuur om TEG standaard op te nemen in het behandelalgoritme van massaal bloedverlies, zoals bediscussieerd is in *Hoofdstuk 2*. Een in 2011 verschenen Cochrane review concludeerde eveneens dat er nog onvoldoende bewijsvoering bestaat dat TEG of ROTEM de morbiditeit en mortaliteit verbetert wanneer deze hemostasetest wordt geïncorporeerd in het behandelalgoritme van massaal bloedverlies. Alhoewel een dergelijke toepassing van TEG de hoeveelheid bloedverlies mogelijk wel beperkt, leidt deze echter niet tot afname van andere pregedefinieerde uitkomsten zoals de incidentie van heroperaties

ten gevolge van bloeding, de gemiddelde opnameduur op de intensive care en het aantal dagen klinische opname.

In een poging de reproduceerbaarheid en standaardisatie van de TEG techniek te verbeteren heeft een internationale groep onderzoekers de handen ineengeslagen en in 2010 de "TEG-ROTEM Working Group" opgericht. In hun eerste studie werd een significante variatie tussen laboratoria gevonden, met variatiecoëfficiënten boven de 10%. Deze studie was overigens de eerste waarbij een serieuze poging werd gedaan om de TEG methodiek te standaardiseren. Het is duidelijk dat er nog aanzienlijke inspanningen geleverd moeten gaan worden om de betrouwbaarheid en reproduceerbaarheid van de techniek verder te verbeteren. In overeenstemming met het verzoek van de Working Group om aanvullend validatie onderzoek te verrichten naar TEG, heeft onze onderzoeksgroep eigen referentiewaarden bepaald in een grote random populatie zoals beschreven in *Hoofdstuk 3*. Tot op heden is dit het eerste onderzoek waarbij in gezonde vrijwilligers met een evenredige verdeling in leeftijd en geslacht zowel klassieke als "nieuwe" TEG parameters zijn bepaald en vergeleken met klassieke stollingstesten, zowel in volbloed als in citraat-ontstold volbloed. Alhoewel onpraktisch ondersteunt onze studie wel de aanbeveling van de fabrikant dat gebruikers van TEG allereerst eigen referentiewaarden dienen te bepalen voordat zij TEG klinisch gaan toepassen. Daarnaast is het van belang om nogmaals te benadrukken dat TEG metingen verricht in volbloed niet uitwisselbaar zijn met die verricht in citraat-ontstold bloed. Verder hebben we aangetoond dat leeftijd, geslacht en gebruik van orale anticonceptiva variabelen zijn die de stollingsstatus duidelijk verhogen. Dit onderstreept nogmaals dat TEG sensitiever is dan de klassieke stollingstesten in het detecteren van verschillen in het hemostatische profiel van patiënten. Alhoewel de "pro-coagulante" effecten van leeftijd en vrouwelijk geslacht subtiel en mogelijk zelfs fysiologisch waren, lijkt het zinvol dat onderzoekers en clinici die gebruik maken van TEG zich bewust zijn van deze effecten bij het interpreteren van testuitslagen.

In operatiekamers wordt TEG in toenemende mate ingezet bij de interpretatie en de hemostatische behandeling van stollingsstoornissen. Een praktisch voordeel van de

techniek is immers dat vrijwel direct aan het bed van de patiënt een snelle en globale indruk kan worden verkregen van diens stollingsstatus en dat direct een hemostatische behandeling kan worden ingezet op basis van het waargenomen TEG profiel en de TEG parameters. Een tekort aan bloedplaatjes (< 50 x 10^9/L) geeft een verhoogd risico op bloedingcomplicaties. Alhoewel TEG parameter Maximale Amplitude (MA) voornamelijk een resultante is van functie en aantallen bloedplaatjes, vonden wij dat deze parameter toch weinig sensitief was indien bij patiënten sprake was van sterk verlaagde bloedplaatjesaantallen, zoals beschreven in *Hoofdstuk 4*. Het meest van belang hierbij is dat in het geval van diepe trombopenie (< 50 x 10^9/L) TEG verricht in volbloed in meer dan 40% van de analyses een volkomen vlakke (inconclusieve) curve opleverde. Tevens was de trombopenie niet alleen van invloed op de MA parameter maar op alle andere TEG parameters. Het is om deze reden dan ook riskant om bij diepe trombopenie het TEG profiel en de TEG parameters (bepaald in volbloed) te gebruiken als leidraad voor hemostatische therapie. Het is daarom noodzakelijk dat voor een correct gebruik en interpretatie van TEG allereerst het effect van andere geïsoleerde stollingsdefecten als ook die van hemostatica worden geëvalueerd en gevalideerd, voordat een op TEG gebaseerd transfusiealgoritme in de analyse en behandeling van bloedverlies kan worden geïncorporeerd.

Transfusie van bloedplaatjes wordt volgens afspraak verricht indien er sprake is van een bloedingsneiging in het kader van een kwalitatieve of kwantitatieve aandoening van de bloedplaatjes of profylactisch indien het risico op bloedingen fors verhoogd is. Optimaal hebben getransfundeerde bloedplaatjes dezelfde hemostatische capaciteit als de natieve circulerende bloedplaatjes. Alhoewel bloedplaatjes producten *in vitro* worden getest op kwaliteit, geven deze testen geen informatie over het *in vivo* hemostatisch potentieel van deze bloedplaatjes. In *Hoofdstuk 5* beschrijven we het gebruik van TEG bij het evalueren van het hemostatische effect van een bloedplaatjestransfusie. Middels TEG konden wij geen verschil aantonen in hemostatische eigenschappen tussen natieve circulerende en getransfundeerde opgeslagen bloedplaatjes. Daarnaast suggereerden onze data dat beperkte opslagduur van bloedplaatjesproducten geassocieerd is met een betere hemostatische capaciteit gedurende tenminste de eerste 24 uur na transfusie. Dit laatste kan klinisch relevant

zijn indien sprake is van een acute bloeding waarbij transfusie van verse bloedplaatjes mogelijk de voorkeur verdient. Bloedplaatjes met langere opslagduur daarentegen kunnen gereserveerd worden voor die gevallen waarin de transfusie profylactisch van aard is. Al met al lijkt deze aanbeveling nog prematuur omdat in onze studie niet het effect op harde eindpunten zoals bloedingsepisoden is meegenomen. Om deze reden zijn toekomstige studies dan ook onontbeerlijk waarbij de middels TEG gemeten hemostatische capaciteit van verse en opgeslagen bloedplaatjes wordt gerelateerd aan het aantal bloedingscomplicaties dat optreedt na transfusie.

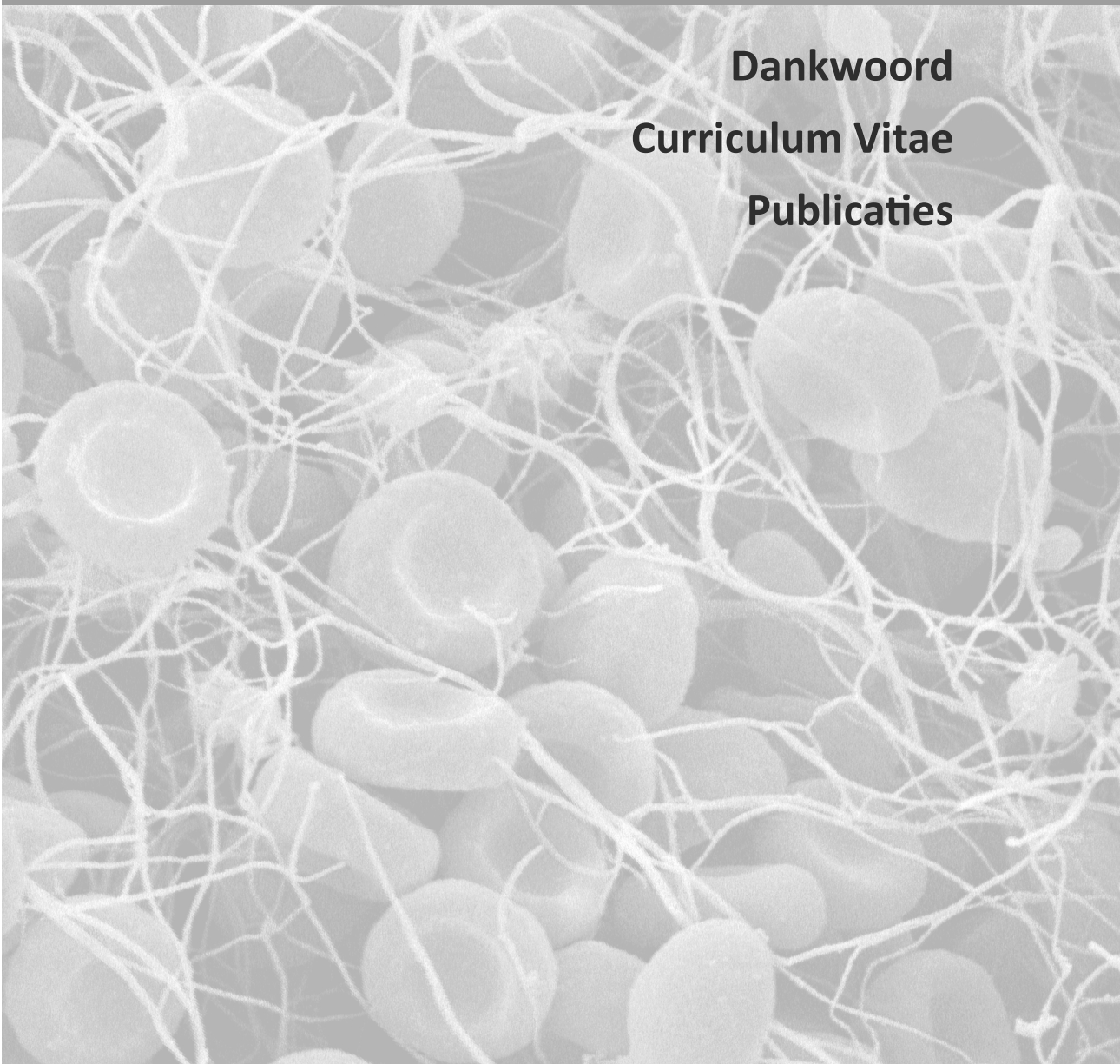
Alhoewel het primaire doel van een transfusie met rode bloedcellen (RBC) het verbeteren van de zuurstoftoevoer naar de weefsels is, hebben RBC ook effecten op de hemostase zoals beschreven is in *Hoofdstuk 6* en *7*. Zo toonden wij aan dat anemie geassocieerd is met een vertraging in de initiatie van de stolling waarbij het uiteindelijk gevormde stolsel wel van een superieure kwaliteit is. Interessant is dat transfusie met RBC exact het tegenovergestelde effect op hemostase sorteerde, met snellere initiatie van de stolling ten koste van de kwaliteit van het stolsel. Opmerkelijk was dat transfusie van verse RBC met een opslagduur van 2 dagen de initiatie van de stolling niet katalyseerde, terwijl wel de kwaliteit van het stolsel nadelig werd beïnvloed. Het lijkt dat beide geobserveerde fenomenen toch verklaard kunnen worden. Allereerst is bekend dat RBC de initiatie van stolling positief beïnvloeden doordat in de bloedstroom deze cellen naar centraal worden gemanoeuvreerd waarbij de kleinere bloedplaatjes hogere concentraties bereiken langs de vaatwand. Ter hoogte van het vaatendotheel vindt vervolgens adhesie, activatie en aggregatie plaats in het kader van de primaire hemostase. Het negatieve effect van RBC op de kwaliteit van het stolsel wordt mogelijk veroorzaakt doordat bij hogere concentratie van RBC deze cellen meer worden ingebouwd in het fibrinenetwerk wat aanleiding geeft tot een minder hechte structuur van het gevormde stolsel. Onze studie-uitkomsten werden recent bevestigd in een *in vitro* studie waarbij hogere concentraties van RBC eveneens gepaard gingen met inferieure kwaliteit van het stolsel. Onze bevindingen zijn relevant voor onderzoekers en clinici actief op het terrein van de hemostase daar zij zich bewust dienen te zijn van de effecten van hematocriet op hemostase parameters bij het interpreteren van TEG parameters. Daarnaast kunnen we slechts

speculeren over een eventuele relatie tussen de hemostatische effecten van RBC en de inferieure klinische uitkomst in kritisch zieke patiënten in relatie tot het aantal bloedtransfusies dat deze patiënten hebben ontvangen. Het belang van RBC in hemostase wordt in *Hoofdstuk 8* nogmaals belicht, waar we TEG gebruikten om hemostase in patiënten met sikkelcelziekte te bestuderen. Alhoewel de verhoogde stollingsstatus bij deze patiënten als multifactorieel wordt beschouwd, bestaat er geen unieke test om deze conditie te kwantificeren of te kwalificeren. Met behulp van TEG waren wij in staat om deze verhoogde stollingsstatus te visualiseren en te kwantificeren, waarbij ten tijde van een pijnlijke sikkelcrisis deze stollingstatus nog verder was toegenomen. Daarnaast waren we in staat om middels TEG de positieve effecten van behandeling met HU op deze stollingstatus aan te tonen. Het blijft overigens nog onzeker of de geobserveerde hemostatische veranderingen causaal zijn aan ziektespecifieke complicaties of dat deze veranderingen eenvoudigweg epifenomenen zijn. Van belang is dat toekomstige studies de relatie tussen TEG parameters, klinische uitkomst en gebruik van HU verder bestuderen.

Wij beschouwen TEG als een veelbelovende techniek voor onderzoek van hemostase, zowel in de klinische als in de experimentele setting, omdat door middel van deze techniek op een unieke en meer fysiologische manier naar stolling kan worden gekeken. In tegenstelling tot klassieke stollingstesten en ondanks het globale karakter van de test is TEG goed in staat om de effecten van leeftijd, geslacht en OAC op het stollingsprofiel weer te geven. Verder is TEG in staat gebleken om de effecten van zowel bloedplaatjes als RBC op hemostase aan te tonen en heeft potentieel als een *in vivo* kwaliteitstest van bloedproducten. Bovendien bestaan er voor TEG toekomstige mogelijkheden op het terrein van analyse en behandeling van patiënten met sikkelcelziekte. Echter ondanks de aanzienlijke mogelijkheden heeft de TEG techniek nog altijd een aantal beperkingen. In de afgelopen jaren is forse progressie geboekt wat betreft de reproduceerbaarheid, betrouwbaarheid, standaardisatie en validatie van de verschillende modificaties en toepassingen van TEG. Desalniettemin kan de TEG techniek nog niet ten volle uit worden benut en toegepast aangezien verrichte studies nog altijd inconsistente uitkomsten opleveren. Het is van groot belang dat met name de klinische voordelen die te behalen zijn met TEG goed

worden gedocumenteerd alvorens de TEG techniek op grote schaal zijn toepassing kan vinden op onder andere de operatiekamer. Verder willen we nogmaals het belang van het verkrijgen van eigen referentiewaarden benadrukken om deze te gebruiken als interne vergelijking. Daarnaast is het vanuit het oogpunt van kwaliteitsbewaking niet minder belangrijk voor TEG dan voor klassieke stollingstesten die in het centrale laboratorium worden bepaald om zowel interne kwaliteitscontrole als externe kwaliteitsbeoordeling te ontwikkelen. Wij hopen dat in de nabije toekomst nog meer geïnvesteerd gaat worden in zowel klinische als experimentele studies met TEG om de diverse toepassingen verder te verbeteren en te stimuleren. Doelgerichte inzet van de TEG techniek, met interpretatie van de TEG curve en haar parameters in de klinische context en gecombineerd met informatie uit klassieke stollingstesten, heeft toegevoegde waarde bij analyse van hemostase bij toekomstige patiënten.

9



Dankwoord

Curriculum Vitae

Publicaties

DANKWOORD

Ik ben gestart met mijn promotieonderzoek als fellow op de afdeling hematologie van het UMCG. Het analyseren en opschrijven van de data heeft uiteindelijk plaatsgevonden in de afgelopen jaren naast mijn werkzaamheden als internist-hematoloog in Ziekenhuis Bethesda te Hoogeveen. Gedurende zowel mijn academische als perifere jaren ben ik een groot aantal personen zeer erkentelijk voor de ontvangen steun, collegialiteit en vooral warmte en plezier waarmee zij mij hebben omringd. Mede dankzij hun motiverende woorden en ondersteuning is dit proefschrift tot stand gekomen. Ik wil dan ook iedereen die op enige wijze hieraan heeft bijgedragen heel hartelijk danken. Daarbij wil ik een aantal personen in het bijzonder noemen.

Geachte prof. dr. J.C. Kluin-Nelemans, beste promotor, beste Hanneke. Nog altijd ben ik je dankbaar voor het feit dat je mij hebt opgeleid in het mooiste deelgebied dat er bestaat binnen de interne geneeskunde, de hematologie. Met plezier en trots kijk ik terug op de fijne jaren die ik op de afdeling Hematologie van het UMCG mocht doorbrengen. Jouw gedrevenheid en enthousiasme om mij - en andere fellows - de fijne kneepjes van het vak bij te brengen heeft nog altijd mijn grote bewondering. Met name je enorme kennis betreffende de morfologie en immunofenotypering van alle denkbare lymfomen en leukemieën hebben op mij een onuitwisbare indruk gemaakt. Ook je voortvarendheid en doortastendheid waarmee je het promotieonderzoek begeleidde zijn voor mij van grote waarde geweest. Ondanks je werkdruk zag je altijd kans om binnen een mum van tijd artikelen, voorzien van waardevol commentaar, te retourneren. Nogmaals mij grote dank hiervoor.

Geachte dr. J.T.M. de Wolf, beste copromotor, beste Joost. Ik ken geen hematoloog die naast zo'n brede hematologische kennis ook nog eens zoveel diepgang per deelgebied kan aanbrengen. Jij hebt me destijds enthousiast gemaakt voor het verrichten van wetenschappelijk onderzoek met de thromboelastografie, wat uiteindelijk heeft geresulteerd in dit proefschrift. Ik heb je niet alleen leren kennen en waarderen als wetenschapper, met een scherp analytisch denkvermogen, maar

ook als positieve en enthousiaste persoonlijkheid. Jaloers ben ik op je indrukwekkend idee- en hypothesegenererend vermogen. Wetenschappelijke discussies met jou waren leerzaam en inspirerend en vonden altijd plaats in een zeer prettige open sfeer, waar ook ruimte was voor humor en “social” talk. Je hebt me gevormd als wetenschapper en daar ben ik je erg erkentelijk voor.

Beste drs. Bosman, beste Lotte. Ik ben je nog altijd dankbaar voor het feit dat je destijds je wetenschappelijke stage op de afdeling Hematologie van het UMCG hebt gedaan. Mede dankzij jouw ijver en doorzettingsvermogen is het ons gelukt om al die honderden bloedmonsters bij patiënten en controlepersonen af te nemen. Ook het verrichten van TEG analyses (vaak buiten kantooruren) was bij jou altijd in vertrouwde handen. Ik kon me geen betere assistent wensen!

Tevens ben ik dr. N.J.G.M Veeger erkentelijk voor zijn waardevolle bijdrage in de statistische onderbouwing van de studie-uitkomsten.

Prof. dr. A. Brand, prof. dr. M.M.R.F. Struys en prof. dr. P.C. Limburg wil ik hartelijk danken voor de uiteindelijke beoordeling van het proefschrift.

Ook wil ik de (toenmalige) stafleden hematologie van het UMCG, in het bijzonder prof. dr. E. Vellenga, prof. dr. J. v.d. Meer (†), dr. S.M.G.J. Daenen en dr. G.W. van Imhoff, bedanken voor hun aandeel in mijn opleiding tot hematoloog. De samenwerking met jullie was uitermate prettig en leerzaam. Ook heden nog maak ik dankbaar gebruik van de waardevolle adviezen inzake hematologische casuïstiek gegeven door prof. dr. E. Vellenga, IKN consulent voor Ziekenhuis Bethesda. Onmisbaar en uitermate leerzaam!

Natuurlijk ben ik ook mijn collega’s van de vakgroep interne geneeskunde van Ziekenhuis Bethesda dank verschuldigd voor de fijne jaren waarin ik met hen het vak interne geneeskunde heb mogen uitoefenen. In het bijzonder ben ik jullie erkentelijk voor de ervaren collegialiteit en amicaliteit. Zonder deze open en warme sfeer, waarin als vanzelfsprekend ruimte bestond voor het verrichten van wetenschappelijk

onderzoek, had mijn promotieonderzoek nooit kunnen gedijen. Met name wil ik hierbij mijn collega dr. F.C. Huvers nog noemen, die “achter de schermen” fungeerde als katalysator. Beste Frank, het zijn jouw enthousiasmerende woorden geweest die er mede voor gezorgd hebben dat ik goed gemotiveerd mijn onderzoek heb kunnen afronden. Ook wil ik je danken voor de geleverde ideeën en kritieken op prille versies van mijn manuscripten. Ik heb grote waardering voor de vanzelfsprekendheid en de belangeloosheid waarmee dit alles gepaard ging!

Tenslotte wil ik natuurlijk mijn directe familie bedanken voor de ervaren steun, warmte en liefde waarmee ik door hen wordt omringd en die o zo belangrijk zijn om te floreren. Uit het diepst van mijn hart wil ik mijn beide ouders bedanken. Lieve pa en ma, alleen dankzij de solide basis die jullie onder mij hebben gelegd en de stimulerende en motiverende woorden die ik van jongs af aan van jullie mocht ontvangen ben ik wat ik nu ben. Lieve Ingrid, jij bent de afgelopen jaren mijn (t)rots in de branding geweest, de veilige haven waarnaar ik altijd kon terugkeren. Zonder jouw onvoorwaardelijke steun en liefde had ik nooit de rust en ruimte kunnen vinden om het promotieonderzoek af te ronden. Daarnaast wist je altijd de balans te bewaken tussen privé en werk zodat we kunnen terugkijken op al heel wat geïncasseerde “quality time” met ons gezin. Onze lieve dochters Jasmijn, Merel en Amber maken ons geluk hierbij compleet.

CURRICULUM VITAE

Wilfried Roeloffzen werd geboren op 8 mei 1970 te Wierden in Nederland. In 1988 behaalde hij het VWO-diploma aan het Pius X college te Almelo. In datzelfde jaar startte hij met de studie geneeskunde aan de Katholieke Universiteit Nijmegen. Het doctoraal examen werd in 1992 met goed gevolg door hem afgelegd. Na een extra-curriculaire stage tropengeneeskunde in Tanzania behaalde hij in 1995 het artsexamen. Omdat zijn interesse was gewekt in de Interne geneeskunde, was hij vervolgens een jaar werkzaam als AGNIO inwendige geneeskunde in het Twenteborg Ziekenhuis te Almelo. In datzelfde ziekenhuis begon hij in 1996 de opleiding tot internist (opleider dr. J. Wolthuis). Vanaf 1998 vervolgde hij zijn opleiding in het Universitair Medische Centrum Groningen (opleider prof. dr. R.O.B. Gans) waarna zijn registratie als internist volgde in 2002. In datzelfde jaar begon hij met de opleiding tot hematoloog, eveneens in het Universitair Medisch Centrum Groningen (opleider prof. dr. J.C. Kluin-Nelemans). Tijdens deze vervolgopleiding tot hematoloog startte hij zijn promotieonderzoek naar de rol van tromboelastografie bij stollingsonderzoek. Zijn aantekening in het deelgebied hematologie behaalde hij in 2005. Vanaf augustus 2005 tot heden is hij als internist hematoloog verbonden aan Ziekenhuis Bethesda te Hoogeveen. Naast zijn klinische werkzaamheden is hij sinds 4 jaar vicevoorzitter van de vereniging medische staf van Ziekenhuis Bethesda, met als portefeuille kwaliteit en veiligheid van zorg. Wilfried Roeloffzen is gehuwd met Ingrid Roeloffzen-Zwijnenberg; samen hebben ze drie dochters Jasmijn, Merel en Amber.

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